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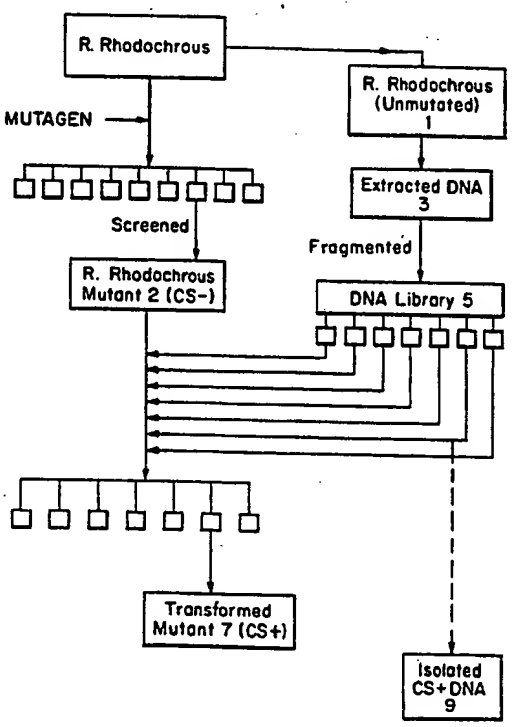
(54) Title: RECOMBINANT DNA ENCODING A DESULFURIZATION BIOCATALYST

(57) Abstract

This invention relates to a recombinant DNA molecule containing a gene or genes which encode a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules. For example, the present invention encompasses a recombinant DNA molecule containing a gene or genes of a strain of *Rhodococcus rhodochrous*.



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RECOMBINANT DNA ENCODING A DESULFURIZATION BIOCATALYST

BACKGROUND

Sulfur contaminants in fossil fuels can create problems in refinery processes which can be costly to rectify. The sulfur contaminants that occur in fossil fuels fall into either of the following general classes: mineralized (inorganic, e.g., pyritic) sulfur and organic sulfur (sulfur that is covalently bound to carbonaceous molecules, referred to as organosulfur compounds). The presence of sulfur has been correlated with corrosion of pipeline, pumping and refining equipment, and with premature breakdown of combustion engines. Sulfur also poisons many catalysts which are used in the refining of fossil fuels. Moreover, the atmospheric emission of sulfur combustion products, such as sulfur dioxide, leads to the form of acid deposition known as acid rain. Acid rain has lasting deleterious effects on aquatic and forest ecosystems, as well as on agricultural areas located downwind of combustion facilities. Monticello, D.J. and W.R. Finnerty, (1985) Ann. Rev. Microbiol. 39:371-389. Regulations such as the Clean Air Act of 1964 require the removal of sulfur, either pre- or post-combustion, from virtually all coal- and petroleum-based fuels. Conformity with such legislation has become increasingly problematic due to the rising need to utilize lower grade, higher-sulfur fossil fuels as clean-burning, low-sulfur petroleum reserves become depleted, as well as the progressive reductions in sulfur emissions required by regulatory authorities. Monticello, D.J. and J.J. Kilbane, "Practical Considerations in Biodesulfurization of Petroleum", IGT's 3d Intl. Symp. on Gas, Oil, Coal, and Env. Biotech., (Dec. 3-5, 1990) New Orleans, LA.

One t chnique which is currently employed for the pre-combustion removal of organic sulfur from liquid

fossil fuels, e.g., petroleum, is hydrodesulfurization (HDS). HDS is suitable for the desulfurization of fossil fuels wherein organosulfur compounds account for a significant, e.g., a major, proportion of all sulfur contaminants present. HDS is thus useful for treating crude oil or bitumen, petroleum distillate fractions or refining intermediates, liquid motor fuels, and the like. HDS is more particularly described in Shih, S.S. et al., "Deep Desulfurization of Distillate Components", Abstract No. 264B AIChE Chicago Annual Meeting, presented November 12, 1990, (complete text available upon request from the American Institute of Chemical Engineers); Gary, J.H. and G.E. Handwerk, (1975) Petroleum Refining: Technology and Economics, Marcel Dekker, Inc., New York, pp. 114-120, and Speight, J.G., (1981) The Desulfurization of Heavy Oils and Residue, Marcel Dekker, Inc., New York, pp. 119-127. HDS is based on the reductive conversion of organic sulfur into hydrogen sulfide (H_2S) in the presence of a metal catalyst. HDS is carried out under conditions of elevated temperature and pressure. The hydrogen sulfide produced as a result of HDS is a corrosive gaseous substance, which is stripped from the fossil fuel by known techniques. Elevated or persistent levels of hydrogen sulfide are known to poison (inactivate) the HDS catalyst, complicating the desulfurization of liquid fossil fuels that are high in sulfur.

Organic sulfur in both coal and petroleum fossil fuels is present in a myriad of compounds, some of which are termed labile in that they can readily be desulfurized, others of which are termed refractory in that they do not easily yield to conventional desulfurization treatment, e.g., by HDS. Shih, S.S. et al. Frequently, then, even HDS-treated fossil fuels must be post-combustively desulfurized using an apparatus such as a flue scrubber. Flue scrubbers are expensive to install and difficult to maintain, especially for small combustion facilities.

Moreover, of the sulfur-generated problems noted above, the use of flue scrubbers in conjunction with HDS is directed to addressing environmental acid deposition, rather than other sulfur-associated problems, such as corrosion of machinery and poisoning of catalysts.

Recognizing these and other shortcomings of HDS, many investigators have pursued the development of microbial desulfurization (MDS). MDS is generally described as the harnessing of metabolic processes of suitable bacteria to the desulfurization of fossil fuels. Thus, MDS typically involves mild (e.g., ambient or physiological) conditions, and does not involve the extremes of temperature and pressure required for HDS. It is also generally considered advantageous that biological desulfurizing agents can renew or replenish themselves under suitable conditions. Microbial desulfurization technology is reviewed in Monticello and Finnerty (1985), 39 ANN. REV. MICROBIOL. 371-389 and Bhadra et al. (1987), 5 BIOTECH. ADV. 1-27. Hartdegan et al. (1984), 5 CHEM. ENG. PROGRESS 63-67 and Kilbane (1989), 7 TRENDS BIOTECHNOL. (No. 4) 97-101 provide additional commentary on developments in the field.

Several investigators have reported mutagenizing naturally-occurring bacteria into mutant strains with the acquired capability of breaking down, i.e., catabolizing, dibenzothiophene (DBT). Hartdegan, F.J. et al., (May 1984) Chem. Eng. Progress 63-67. DBT is representative of the class of organic sulfur molecules found in fossil fuels from which it is most difficult to remove sulfur by HDS. Most of the reported mutant microorganisms act upon DBT nonspecifically, by cleaving carbon-carbon bonds, thereby releasing sulfur in the form of small organic breakdown products. One consequence of this microbial action is that the fuel value of a fossil fuel so treated is degraded. Isbister and Doyle, however, reported the derivation of a mutant strain of Pseudomonas which appeared to be capable of selectively liberating sulfur from

DBT, thereby preserving the fuel value of treated fossil fuels. U.S. Patent No. 4,562,156.

Kilbane recently reported the mutagenesis of a mixed bacterial culture, producing a bacterial consortium which appeared capable of selectively liberating sulfur from DBT by an oxidative pathway. Resour. Cons. Recycl. 3:69-79 (1990). A strain of Rhodococcus rhodocrous was subsequently isolated from the consortium. This strain, which has been deposited with the American Type Culture Collection under the terms of the Budapest Treaty as ATCC No. 53968 and also referred to as IGTS8, is a source of biocatalytic activity as described herein. Microorganisms of the ATCC No. 53968 strain liberate sulfur from forms of organic sulfur known to be present in fossil fuels, including DBT, by the selective, oxidative cleavage of carbon-sulfur bonds in organic sulfur molecules. Kilbane has described the isolation and characteristics of this strain in detail in U.S. Patent No. 5,104,801.

SUMMARY OF THE INVENTION

This invention relates in one aspect to a deoxyribonucleic acid (DNA) molecule containing one or more genes encoding one or more enzymes that, singly or in concert with each other, act as a biocatalyst that desulfurizes a fossil fuel that contains organic sulfur molecules. The DNA molecule of the present invention can be purified and isolated from a natural source, or can be a fragment or portion of a recombinant DNA molecule that is, e.g., integrated into the genome of a non-human host organism. The gene or genes of the present invention can be obtained from, e.g., a strain of Rhodococcus rhodochrous microorganisms having suitable biocatalytic activity. That is, the gene or genes of the present invention can be obtained from a non-human organism, e.g., a microorganism, that expresses one or more enzymes that, singly or in concert with each other, act as a desulfurizing biocatalyst.

Biocatalysis, as described more fully below, is the selective oxidative cleavage of carbon-sulfur bonds in organosulfur compounds. The present invention is particularly useful for the desulfurization of fossil fuels that contain organosulfur compounds, e.g., DBT.

The invention further relates to recombinant DNA vectors, recombinant DNA plasmids and non-human organisms that contain foreign (recombinant, heterologous) DNA encoding a biocatalyst capable of desulfurizing a fossil fuel which contains organosulfur compounds. Such organisms are referred to herein as host organisms.

The invention described herein thus encompasses ribonucleic acid (RNA) transcripts of the gene or genes of the present invention, as well as polypeptide expression product(s) of the gene or genes of the present invention. The present polypeptide expression products, after such post-translational processing and/or folding as is necessary, and in conjunction with any coenzymes, cofactors or coreactants as are necessary, form one or more protein biocatalysts (enzymes) that, singly or in concert with each other, catalyze (promote, direct or facilitate) the removal of sulfur from organosulfur compounds that are found in fossil fuels. This is accomplished by the selective, oxidative cleavage of carbon-sulfur bonds in said compounds. The biocatalyst of the present invention can be a non-human host organism, viable (e.g., cultured) or non-viable (e.g., heat-killed) containing the DNA of the present invention and expressing one or more enzymes encoded therein, or it can be a cell-free preparation derived from said organism and containing said one or more biocatalytic enzymes.

In another aspect, the present invention relates to a method of desulfurizing a fossil fuel using the above mentioned non-human organism, said organism expressing a desulfurizing biocatalyst. Alternativ ly, the pr sent invention relates to a method of desulfurizing a fossil

fuel using a biocatalyst preparation comprising one or more enzymes isolated from said organism. The process involves: 1) contacting said organism or biocatalyst preparation obtained therefrom with a fossil fuel that contains organic sulfur, such that a mixture is formed; and 2) incubating the mixture for a sufficient time for the biocatalyst expressed by or prepared from the organism to desulfurize the fossil fuel. The biocatalytically treated fossil fuel obtained following incubation has significantly reduced levels of organosulfur compounds, compared to a sample of the corresponding untreated fossil fuel.

In yet another aspect, the invention relates to nucleic acid probes which hybridize to the recombinant DNA of the present invention.

In still other aspects, the present invention relates to the production of new non-human organisms containing the recombinant DNA of the present invention and preferably expressing the biocatalyst encoded therein. Availability of the recombinant DNA of this invention greatly simplifies and facilitates the production and purification of biocatalysts for desulfurizing a fossil fuel. Costly and time consuming procedures involved in the purification of biocatalysts can be reduced, eliminating the need to generate the biocatalyst from one or more non-human organisms in which it is naturally present or has been produced by mutagenesis. More specifically, non-human host organisms can be generated which express the gene or genes of the present invention at elevated levels. In addition, the invention described herein furthers the discovery of genes encoding desulfurization biocatalysts in additional non-human organisms. This objective can be accomplished using the nucleic acid probes of the present invention to scre n DNA libraries prepared from one or more additional non-human organisms in whom biocatalytic function is known or susp cted to be pr sent. Any genes present in such

organisms and encoding desulfurization biocatalysts or components thereof can be replicated at large scale using known techniques, such as polymerase chain reaction (PCR). PCR advantageously eliminates the need to grow the non-human organisms, e.g., in culture, in order to obtain large amounts of the DNA of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow diagram schematic illusting a stepwise procedure for the isolation of the recombinant DNA of the present invention.

Figure 2 is a schematic illustration of the Rhodococcus rhodochrous replication competent and chloramphenicol resistant vector pRF29, said vector having been derived from Rhodococcus fascians.

Figure 3 is a schematic illustration of the Rhodococcus rhodochrous replication competent and chloramphenicol resistant vector pRR-6.

Figure 4 is a schematic illustration of the restriction map for DNA plasmid pTOXI-1 encoding a biocatalyst capable of carbon-sulfur bond cleavage.

Figure 5 is a schematic illustration of the restriction map for subclone pMELV-1, derived from plasmid pTOXI-1.

Figure 6 is a schematic illustration of the restriction map for pMELV-1 and fragments thereof present as inserts in subclones pSMELV-1A, pSMELV-2A, pSMELV-3A and pSMELV-4A.

Figure 7 is a schematic illustration of the predicted locations within the sequence of pTOXI-1 of three nearly contiguous open reading frames (ORFs; specifically, ORF 1, ORF 2 and ORF 3) encoding polypeptide expression products responsible for the Dsz+ phenotype.

Figure 8 is a schematic illustration of the restriction map of pTOXI-1 and fragments thereof present as inserts in subclones pENOK-1, pENOK-2, pENOK-3, pENOK-11,

pENOK-13, pENOK-16, pENOK-18, pENOK-Nsi, pENOK-19 AND pENOK-20.

Figure 9 is a schematic illustration of the restriction map of pRR-12.

Figure 10 is a schematic illustration of the restriction map of vector pKAMI. In the inset, the engineered cloning site present in pKAMI is shown in detail.

Figure 11 is a schematic illustration of the restriction map of pSBG-2, in which expression of a promoterless Dsz gene cluster from pTOXI-1 is driven by the chloramphenicol resistance promoter.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the petroleum extraction and refining arts, the term "organic sulfur" is generally understood as referring to organic molecules having a hydrocarbon framework to which one or more sulfur atoms (called heteroatoms) are covalently joined. These sulfur atoms can be joined directly to the hydrocarbon framework, e.g., by one or more carbon-sulfur bonds, or can be present in a substituent joined to the hydrocarbon framework of the molecule, e.g., a sulfonyl group (which contains a carbon-oxygen-sulfur covalent linkage). The general class of organic molecules having one or more sulfur heteroatoms are sometimes referred to as "organosulfur compounds". The hydrocarbon portion of these compounds can be aliphatic, aromatic, or partially aliphatic and partially aromatic.

Cyclic or condensed multicyclic organosulfur compounds in which one or more sulfur heteroatoms are linked to adjacent carbon atoms in the hydrocarbon framework by aromatic carbon-sulfur bonds are referred to as "sulfur-bearing heterocycles". The sulfur that is present in many types of sulfur-bearing heterocycles is referred to as "thiophenic sulfur" in vi w of the five-membered aromatic ring in which the sulfur heteroatom is present. The

simplest such sulfur-bearing heterocycle is thiophene, which has the composition C_4H_4S.

Sulfur-bearing heterocycles are known to be stable to conventional desulfurization treatments, such as HDS. For this reason, they are said to be refractory or recalcitrant to HDS treatment. Sulfur-bearing heterocycles can have relatively simple or relatively complex chemical structures. In complex heterocycles, multiple condensed aromatic rings, one or more of which can be heterocyclic, are present. The difficulty of desulfurization increases with the structural complexity of the molecule. Shih et al. That is, refractory behavior is most accentuated in complex sulfur-bearing heterocycles, such as dibenzothiophene (DBT, $C_{12}H_8S$).

DBT is a sulfur-bearing heterocycle that has a condensed, multiple aromatic ring structure in which a five-membered thiophenic ring is flanked by two six-membered benzylic rings. Much of the residual post-HDS organic sulfur in fossil fuel refining intermediates and combustible products is thiophenic sulfur. The majority of this residual thiophenic sulfur is present in DBT and derivatives thereof having one or more alkyl or aryl radicals attached to one or more carbon atoms present in one or both flanking benzylic rings. Such DBT derivatives are said to be "decorated" with these radicals. DBT itself is accepted in the relevant arts as a model compound illustrative of the behavior of the class of compounds encompassing DBT and alkyl- and/or aryl-decorated derivatives thereof in reactions involving thiophenic sulfur. Monticello and Finnerty (1985), Microbial desulfurization of fossil fuels, 39 ANNUAL REVIEWS IN MICROBIOLOGY 371-389, at 372-373. DBT and radical-decorated derivatives thereof can account for a significant percentage of the total sulfur content of particular crude oils, coals and bitumen. For example, these sulfur-bearing heterocycles have been reported to account for as much

as 70 wt% of the total sulfur content of West Texas crude oil, and up to 40 wt% of the total sulfur content of some Middle East crude oils. Thus, DBT is considered to be particularly relevant as a model compound for the forms of thiophenic sulfur found in fossil fuels, such as crude oils, coals or bitumen of particular geographic origin, and various refining intermediates and fuel products manufactured therefrom. Id. Another characteristic of DBT and radical-decorated derivatives thereof is that, following a release of fossil fuel into the environment, these sulfur-bearing heterocycles persist for long periods of time without significant biodegradation. Gundlach et al. (1983), 221 SCIENCE 122-129. Thus, most prevalent naturally occuring microorganisms do not effectively metabolize and break down sulfur-bearing heterocycles.

A fossil fuel that is suitable for desulfurization treatment according to the present invention is one that contains organic sulfur. Such a fossil fuel is referred to as a "substrate fossil fuel". Substrate fossil fuels that are rich in thiophenic sulfur (wherein a significant fraction of the total organic sulfur is thiophenic sulfur, present in sulfur-bearing heterocycles, e.g., DBT) are particularly suitable for desulfurization according to the method described herein. Examples of such substrate fossil fuels include Cerro Negro or Orinoco heavy crude oils; Athabascan tar and other types of bitumen; petroleum refining fractions such as light cycle oil, heavy atmospheric gas oil, and No. 1 diesel oil; and coal-derived liquids manufactured from sources such as Pocahontas #3, Lewis-Stock, Australian Glencoe or Wyodak coal.

Biocatalytic desulfurization (biocatalysis or BDS) is the excision (liberation or removal) of sulfur from organosulfur compounds, including refractory organosulfur compounds such as sulfur-bearing heterocycles, as a result of the selective, oxidative cleavage of carbon-sulfur bonds in said compounds by a biocatalyst. BDS treatment

yields the desulfurized combustible hydrocarbon framework of the former refractory organosulfur compound, along with inorganic sulfur -- substances which can be readily separated from each other by known techniques such as fractional distillation or water extraction. For example, DBT is converted into hydroxybiphenyl or dihydroxybiphenyl, or a mixture thereof, when subjected to BDS treatment. BDS is carried out by a biocatalyst comprising one or more non-human organisms (e.g., microorganisms) that functionally express one or more enzymes that direct, singly or in concert with each other, the removal of sulfur from organosulfur compounds, including sulfur-bearing heterocycles, by the selective cleavage of carbon-sulfur bonds in said compounds; one or more enzymes obtained from such microorganisms; or a mixture of such microorganisms and enzymes. Organisms that exhibit biocatalytic activity are referred to herein as being CS+ or Dsz+. Organisms that lack biocatalytic activity are referred to herein as being CS- or Dsz-.

As summarized above, the invention described herein relates in one aspect to a DNA molecule or fragment thereof containing a gene or genes which encode a biocatalyst capable of desulfurizing a fossil fuel that contains organosulfur compounds. The present DNA molecule or fragment thereof can be purified and isolated DNA obtained from, e.g., a natural source, or can be recombinant (heterologous or foreign) DNA that is, e.g., present in a non-human host organism. The following discussion, which is not to be construed as limiting on the invention in any way but is presented for purposes of illustration, recounts the isolation of DNA encoding a desulfurizing biocatalyst from a strain of Rhodococcus rhodochrous, ATCC No. 53968, that is known to express suitable biocatalytic activity. This pref rred strain of Rhodococcus rhodocrous is disclosed in U.S. Patent No. 5,104,801 (issued 1992), the teachings of which are incorporated herein by refer-

ence, and has been referred to in the literature as IGTS8. IGTS8 was developed by investigators at the Institute of Gas Technology in Chicago IL. Other organisms that are known to express suitable biocatalytic activity and thus are viewed as suitable sources of the DNA of the present invention include the strain of Bacillus sulfasportare described in U.S. Patent 5,002,888 and deposited with the American Type Culture Collection as ATCC No. 53969 and the Corynebacterium strain described in Omori et al. (1992), Desulfurization of dibenzothiophene by Corynebacterium sp. strain SY1, 58 APPL. ENV. MICROBIOL. (No. 3) 911-915. The isolation of the DNA of the present invention from the ATCC No. 53968 microorganism is schematically depicted in Figure 1, and will now be described.

Mutant strains of R. rhodochrous that are incapable of cleaving carbon-sulfur bonds (CS- or Dsz-), are produced by exposing a strain of R. rhodochrous, e.g., ATCC No. 53968, that exhibits biocatalytic activity (that is CS+ or Dsz+), to a mutagen under appropriate conditions that are known to or readily ascertainable by those skilled in the art. Suitable mutagens include radiation, e.g., ultraviolet radiation, and chemical mutagens, e.g., N-methyl-N'-nitrosoguanidine (NTG), hydroxylamine, ethylmethanesulphonate (EMS) and nitrous acid. Mutants thus formed are allowed to grow in an appropriate medium and screened for carbon-sulfur bond cleavage activity. Mutants identified as lacking carbon-sulfur bond cleavage activity are termed CS-. Any method of screening which allows for an accurate detection of carbon-sulfur bond cleavage activity is suitable in the method of the present invention. Suitable methods of screening for this activity include exposing the different mutants to carbon-sulfur bond containing molecules (e.g., DBT) and measuring carbon-sulfur bond cleavage. In a preferred embodiment, the mutants are exposed to DBT, such that the breakdown product, hydroxybiphenyl (HBP), which fluoresces under

short wave ultraviolet light, is produced. HBP can also be detected colorimetrically through its blue reaction product with Gibbs' reagent. Other methods include gas and liquid chromatography, infrared and nuclear magnetic resonance spectrometry. See Kodama, et al., Applied and Environmental Microbiology, pages 911-915 (1992) and Kilbane and Bielaga, Final Report D.O.E. Contract No. DE-AC22-88PC8891 (1991). Once CS- mutants are identified and isolated, clones thereof are propagated using standard techniques and subjected to further analysis.

Concurrent with the mutagenesis of the above-described culture of the CS+ organism, R. rhodochrous, a second culture of the same CS+ organism (1) is maintained in culture. CS+ organism DNA (3) is extracted from this culture of R. rhodocrous. Various methods of DNA extraction are suitable for isolating the DNA of this organism. Suitable methods include phenol and chloroform extraction. See Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press, page 16.54 (1989), herein referred to as Maniatis et al..

Once the DNA is extracted from R. rhodochrous 1, the DNA (3) is cut into fragments of various kilobase lengths, which collectively make up DNA library 5. Various methods of fragmenting the DNA of R. rhodochrous to purify DNA therefrom, including the DNA of the present invention, can be used, e.g., enzymatic and mechanical methods. Any four-base recognition restriction endonuclease such as TaqI or Sau 3A is suitable for fragmenting the DNA. Suitable methods of fragmenting DNA can be found in Maniatis et al..

The various DNA fragments are inserted into several CS- mutant clones of R. rhodochrous (2), with the purpose of isolating the fragment of DNA that encodes the biocatalyst of the present invention. The transformation of a previously CS- mutant cell to a CS+ transformed cell is evidence that the inserted DNA fragment encodes a bio-

catalyst. Any method of inserting DNA into R. rhodochrous which allows for the uptake and expression of said fragment is suitable. In a preferred embodiment, electroporation is used to introduce the DNA fragment into R. rhodochrous. See Maniatis et al..

Once transformed, CS+ mutant R. rhodochrous 7 has been produced and identified, DNA fragment 9 encoding the CS+ biocatalyst can be identified and isolated. The encoded biocatalyst can then be produced using the isolated DNA in various methods that are well known and readily available to those skilled in the art. In addition, the isolated DNA can be sequenced and replicated by known techniques, e.g., the techniques described in Maniatis et al..

As noted previously, the above-described method for isolating the DNA of the present invention can be applied to CS+ organisms other than R. rhodocrous microorganisms, e.g., of the strain ATCC No. 53968. Thus, Bacillus sulfasportare ATCC No. 53969 or Corynebacterium sp. SY1 can be used as the source organism for the DNA of the present invention. Furthermore, once isolated, the DNA of the present invention can be transfected into a non-human host organism other than a CS- mutant of the source organism. Thus, the DNA of the present invention can be transfected into, e.g., a suitable strain of Escherichia coli bacteria. Other types of non-human host organism can also be used, including unicellular organisms (e.g., yeast) and cells established in culture from multicellular organisms.

Other methods of isolating the DNA of the present invention, include variations on the rationale described above and depicted in Figure 1. For example, it would be possible to randomly insert a CS- DNA plasmid into clones of a CS+ strain of R. rhodochrous. DNA encoding a CS+ biocatalyst could then be identified by screening for clones that have been transformed from CS+ to CS-.

The recombinant DNA molecule or fragment thereof of the present invention is intended to encompass any DNA resulting from the insertion into its chain, by chemical or biological means, of one or more genes encoding a biocatalyst capable of selectively cleaving carbon-sulfur bonds, said gene not originally present in that chain. Recombinant DNA includes any DNA created by procedures using restriction nucleases, nucleic acid hybridization, DNA cloning, DNA sequencing or any combination of the preceding. Methods of construction can be found in Maniatis et al., and in other methods known by those skilled in the art.

Procedures for the construction of the DNA plasmids or vectors of the present invention include those described in Maniatis et al. and other methods known by those skilled in the art. Suitable plasmid vectors include pRF-29 and pRR-6 depicted in Figures 2 and 3, respectively. The terms "DNA plasmid" and "vector" are intended to encompass any replication competent plasmid or vector capable of having foreign or exogenous DNA inserted into it by chemical or biological means and subsequently, when transfected into an appropriate non-human host organism, of expressing the product of the foreign or exogenous DNA insert (i.e., of expressing the biocatalyst of the present invention). In addition, the plasmid or vector must be receptive to the insertion of a DNA molecule or fragment thereof containing the gene or genes of the present invention, said gene or genes encoding a biocatalyst that selectively cleaves carbon-sulfur bonds in organosulfur compounds. Procedures for the construction of DNA plasmid vectors include those described in Maniatis et al. and others known by those skilled in the art.

The plasmids of the present invention include any DNA fragment containing a gene or genes encoding a biocatalyst that selectively cleaves carbon-sulfur bonds in organosulfur compounds. The term "plasmid" is intended to

encompass any DNA fragment. The DNA fragment should be transmittable to a host microorganism by transformation or conjugation. Procedures for the construction or extraction of DNA plasmids include those described in Maniatis et al. and others known by those skilled in the art.

The transformed non-human host organisms of the present invention can be created by various methods by those skilled in the art. For example, transfection electroporation as explained by Maniatis et al. can be used. By the term "non-human host organism" is intended any non-human organism capable of the uptake and expression of foreign, exogenous or recombinant DNA, i.e., DNA not originally a part of the organism's nuclear material.

The method of desulfurizing a fossil fuel of the present invention involves two aspects. First, a host organism or biocatalytic preparation obtained therefrom is contacted with a fossil fuel to be desulfurized. This can be done in any appropriate container, optionally fitted with an agitation or mixing device. The mixture is combined thoroughly and allowed to incubate for a sufficient time to allow for cleavage of a significant number of carbon-sulfur bonds in organosulfur compounds, thereby producing a desulfurized fossil fuel. In one embodiment, an aqueous emulsion is produced with an aqueous culture of the organism and the fossil fuel, allowing the organism to propagate in the emulsion while the expressed biocatalyst cleaves carbon-sulfur bonds.

Variables such as temperature, mixing rate and rate of desulfurization will vary according to the organism used. The parameters can be determined through no more than routine experimentation.

Several suitable techniques for monitoring the rate and extent of desulfurization are well-known and readily available to those skilled in the art. Baseline and timecourse samples can be collected from the incubation mixture, and prepared for a determination of the residual

organic sulfur in the fossil fuel. The disappearance of sulfur from organosulfur compounds, such as DBT, in the sample being subjected to biocatalytic treatment can be monitored using, e.g., X-ray fluorescence (XRF) or atomic emission spectrometry (flame spectrometry). Preferably, the molecular components of the sample are first separated, e.g., by gas chromatography.

The nucleic acid probes of the present invention include any nuclear material capable of hybridizing to at least a portion of the DNA of the present invention. The term "nucleic acid probe" includes any nuclear material capable of hybridizing to DNA.

The invention will now be further illustrated by the following specific Examples, which are not to be viewed as limiting in any way.

EXAMPLE 1. ISOLATION OF DNA ENCODING A DESULFURIZATION ACTIVE BIOCATALYST.

As used herein, the term "Dsz+" refers to the ability of an organism to utilize thiophenic compounds such as dibenzothiophene (DBT) as the sole source of sulfur by the selective cleavage of carbon-sulfur bonds therein. Rhodococcus rhodochrous strain IGTS8 demonstrates the Dsz$^+$ phenotype. The term "Dsz-" referrs to an organism's inability to utilize said thiophenic compounds as a sole source of sulfur by the selective cleavage of carbon-sulfur bonds therein.

Materials

Bacterial strains and plasmids

Rhodococcus rhodochrous strain IGTS8 (ATCC No. 53968), obtained from the Institute of Gas Technology (Chicago, IL), was used as a parent strain for production of mutant strains which have lost the desulfurization phenotype (Dsz-). Strain IGTS8 was also us d for isolation of DNA fragments capable of complementing said

mutants to produce Dsz+ mutants therefrom. Rhodococcus vector pRF-29 was obtained from the Institute of Gas Technology. The construction of pRF-29 is described in Desomer, et al. (1990), Transformation of Rhodococcus fascians by High-Voltage Electroporation and Development of R. fascians Cloning Vectors, APPLIED AND ENVIRONMENTAL MICROBIOLOGY 2818-2825. The structure of pRF-29 is schematically depicted in Figure 2.

Escherichia coli strain JM109 was used as a host in transformation with plasmid constructs derived from the plasmids pUC18 and pUC19 (Bethesda Research Laboratories, Bethesda, MD).

Enzymes and Reagents

Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) and New England Biolabs (Beverly, MA). T4 ligase and the Klenow fragment of E. coli DNA polymerase I were purchased from BRL. HK™ Phosphatase was purchased from Epicentre Technologies (Madison, WI). All enzymes were used in accordance with manufacturers recommendations. Enzyme assay substrates Dibenzothiophene (DBT), Dibenzothiophene 5-oxide (DBT sulfoxide) and Dibenzothiphene sulfone (DBT sulfone) were purchased from Aldrich (Milwaukee, WI). Gibb's Reagent, 2,6-dicholoroquinone-4-chloroimide, was purchased from Sigma (St. Louis, MO). Chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was also purchased from Sigma.

Growth Media and Conditions

E. coli JM109 was grown in L-broth (Difco, Detroit, MI). Transformants were selected on L-plates supplemented with 1.5% agar and containing 125µg/ml ampicillin. E. coli strains were grown at 37°C. Rhodococcus strains were maintained on Rhodococcus Media (RM) composed per liter of: 8.0g Nutrient Broth (Difco), 0.5g yeast extract, 10.0g

glucose. Transformants of Rhodococcus strains were selected on RM plates supplemented with 1.5% agar and containing 25µg/ml chloramphenicol. For expression of the Dsz+ phenotype, Rhodococcus strains were grown in Basal Salts Media (BSM) composed per liter of: 2.44g KH_2PO_4, 5.57g Na_2HPO_4 2.0g NH_4Cl, 0.2 g $MgCl_2.6H_2O$, 0.001g $CaCl_2.2H_2O$, 0.001g $FeCl_3.6H_2O$, 0.004g $MnCl_2.4H_2O$, 6.4ml glycerol. Optionally, BSM can be supplemented with 5.0g/liter glucose. Rhodococcus strains were grown at 30°C.

Methods

Sulfur Bioavailability Assay

The sulfur bioavailability assay, described in U.S. Patent 5,104,801, examines an organism's ability to liberate organically bound sulfur from substrates (e.g., DBT, DBT sulfoxide, DBT sulfone) for use as the sole source of sulfur for growth. In the assay, BSM, which contains no sulfur, is supplemented with one or more sulfur containing substrates, e.g., DBT. The organism's ability to liberate sulfur therefrom is measured by its ability to grow with proper incubation, as monitored by optical density at 600 nm.

Gibbs Assay for 2-Hydroxybiphenyl

The oxidative product of DBT, DBT sulfoxide and DBT sulfone incubated with strain IGTS8 is 2-hydroxybiphenyl (2-HBP). The Gibbs assay colorimetrically quantitates the amount of 2-HBP produced from DBT and its above-mentioned oxidative derivatives. The assay measures 2-HBP produced in culture supernatants after incubation with DBT. The media must be adjusted to pH 8.0 before the Gibb's reagent is added. Gibb's R agent, 2,6- dicholoroquinone-4-chloroimide (10mg/ml in ethanol), is added to culture supernatants at 1:100 (v/v). Color development is measured as

absorbance at 610nm after a 30 minute incubation at room temperature.

HPLC Assay for 2-Hydroxybiphenyl

2-HBP production cultures incubated with DBT can also be detected by HPLC using instrumentation available from Waters, Millipore Corporation, Milford, MA. Reagent alcohol is added to culture broth at 1:1 (v/v) in order to solubilize all remaining DBT and 2-HBP. Samples are agitated for 5 min at 220 rpm. Extracted broth samples are removed and centrifuged to remove cellular mass. Clarified supernatants are then analyzed by HPLC with the following conditions:

Column:	Waters 4μ Phenyl Novapak	
Detection Parameters:	DBT	233nm, 1.0 AUFS
	2-HBP	248nm, 0.2 AUFS
Quantitative Detection Limits:	DBT	10 - 250 μM
	2-HBP	6 - 60 μM
Mobile Phase:	Isocratic 70% Acetonitrile 1.5ml/min	
Retention times:	DBT	4.5 minutes
	2-HBP	2.9 minutes

IGTS8 Mutagenesis

In order to generate mutant strains of R. rhodochrous which did not metabolize DBT (Dsz- mutants), biocatalyst source strain IGTS8 (Dsz+) was subjected to mutagenesis by short-wave UV light and to chemical mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). With UV exposure mutagenesis, a kill rate of greater than 99% was targeted. Continuously stirred R. rhodochrous cells at an optical density (A_{660}) of 0.3 were subj cted to UV exposure from a Min ralight Lamp Model UVG-254 (Ultra-violet Pro-

ducts, Inc., San Gabriel, CA) at a distance of 10 cm for 55 to 65 seconds to obtain this kill rate (97.9-99.9%). For NTG mutagenesis, cell suspensions were treated with 500 µg/ml NTG for a duration determined to achieve a kill rate of 30%-50%. Combination mutagenesis utilizing both NTG and UV was also done. For these an overall kill rate of greater than 99.9% was used. Colonies surviving mutagenesis were picked onto RM plates and screened for the Dsz- phenotype as described below.

Screening Example A: Initially, a DBT-spray plate screen was used to select Dsz- mutants. Mutant colonies were replica plated onto Basal Salts Media (BSM) electrophoretic-grade agarose plates which contained no added sulfur. Colonies were allowed to grow at 30°C for 24hr. The plates were then sprayed with an even coating of 10% DBT dissolved in ether and incubated at 30°C for 90 minutes. The plates were then wiped clean and observed under short-wave UV light. The observed end product of DBT metabolism, 2-hydroxybiphenyl (2-HBP) fluoresces under short-wave UV light. Colonies that produce 2-HBP are thus identified by fluorescent spots on the agarose. Colonies that do not produce 2-HBP (that are Dsz-) do not produce fluorescent spots.

Screening Example B: A simpler variation of screening involved replica plating surviving mutagenized colonies to BSM agarose plates supplemented with 1.2ml/liter of a saturated ethanol solution of DBT. After 24 hours, production of 2-HBP can be visualized under UV illumination as above.

Mutants which did not appear to produce 2-HBP by the above-described screening methods were examined with the sulfur bioavailability assay, with DBT as the sole source of sulfur. Growth of potential mutants was examined in

1.25ml liquid fermentations in BSM plus DBT media dispensed in 24-well plates (Falcon). After a one day incubation at 30°C, 2-HBP production was monitored by the Gibbs colorimetric assay. Strains which continue to demonstrate the Dsz- phenotype were incubated in larger volumes of BSM plus DBT and analyzed for 2-HBP or intermediates by the HPLC method. Because BSM is a defined minimal medium, a duplicate control culture which contained supplemental inorganic sulfur was grown in order to distinguish true Dsz- mutants from auxotrophic mutants. Mutants which failed to grow in both the control and experimental media were assumed to be auxotrophic mutants.

Of 1970 individually analyzed potential mutants, two were identified as Dsz-. One mutant, GPE-362, was generated by NTG mutagenesis. The other, CPE-648, was generated by combination NTG/UV mutagenesis. Both GPE-362 and CPE-648 grow slowly in sulfur bioavailability assays, presumably from trace amounts of sulfur on the glassware or in the media components. However, no detectable amounts of 2-HBP were produced by either mutant after an extended incubation of 6 to 10 days with DBT, as assessed with either the Gibbs assay or the HPLC assay. Thus, independently produced R. rhodocrous IGTS8 mutants GPE-362 and CPE-648 were Dsz- organisms.

Vector Construction

Vector constructs were derived from R. rhodochrous and confer chloramphenicol resistance. All constructs were developed in E. coli strain JM109. Transformation of JM109 was carried out with the Gene Pulsar (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's recommendations. Plasmid isolation from JM109 was performed by standard methods (Birnboim and Doly (1979), A rapid alkaline extraction procedure for screening recombinant plasmid DNA, 7 NUCLEIC ACIDS RES. 1513-1523; Maniatis et al. (1982), MOLECULAR CLONING: A LABORATORY MANUAL (Cold

Spring Harbor Laboratory Press). Transformants containing correct vector constructs were identified by restriction analysis.

Vector Construct A: pRR-6 (Figure 3) contains the Rhodococcus origin of replication and Chloramphenicol resistance marker (Cm^R). The ori and Cm^R have been removed from pRF-29 as a 6.9kb XhoI/Xba (partial) fragment. The ends were made blunt with Klenow and ligated to SaII/XbaI cut pKF39. pKF39 is pUC18 with the SmaII site replaced with a BgIII site. A unique NarI site is available for cloning in pRR-6. NarI ends are compatible with 4-base recognition endonuclease TaqI.

Transformation of Rhodococcus rhodochrous

Transformation of IGTS8 and Dsz- mutants thereof can be achieved by electroporation. The following conditions were used in all transformations of Rhodococcus rhodochrous. Cells were grown in RM to mid-log phase and harvested by centrifugation (5000xg), then washed three times in cold, deionized, distilled water and concentrated 50-fold in 10% glycerol. The resulting cell concentrate could be used for electroporation directly or stored at -80°C.

Electroporations were carried out with the Gene Pulser (Bio-Rad) apparatus. 100 µl cells were mixed with transformation DNA in a 2-mm gapped electrocuvette (Bio-Rad) and subjected to a 2.5 kV pulse via the pulse controller (25 µF capacitor, 200 Ω external resistance). Pulsed cells were mixed with 400 µ RM and incubated for 4 hours at 30°C with regular agitation. Cells were then plated to RM supplemented with proper antibiotic.

When IGTS8 was transformed with pRF-29, chloramphenicol resistant colonies were cleanly selected at a frequency of 10^5 - 10^6/µg DNA on plates containing 25 µg/ml chloramphenicol.

Small Scale Plasmid Preparation from R. rhodochrous

A single colony of Rhodococcus rhodochrous was used to inoculate 2 to 7ml of RM plus 25 µg/ml chloramphenicol. The culture was incubated for two days at 30°C with shaking. Cells were pelleted by centrifugation and resuspended in 300 µl sucrose buffer (20% sucrose, 0.05 M Tris-Cl pH 8.0, 0.01 M EDTA 0.05 M NaCl, 10 mg/ml lysozyme) and incubated at 37°C for 1 hour. 300 µl Potassium acetate-acetate solution, pH 4.8 (60 ml 5 M KOAc, 11.5 ml Glacial acetic acid, 28.5 ml dH_2O), was added and the mixture was gently mixed by inversion. The mixture was placed on ice for 5 minutes and then cellular debris was pelleted by centrifugation. 500 µl supernatant was removed to a fresh tube to which RNAse was added to 0.05 µg/µl and incubated for 20 minutes at 37°C. The sample was then phenol:-chloroform extracted and the aqueous layer was precipitated at -80°C with an equal volume of isopropanol. DNA was pelleted by centrifugation and resuspended in 0.3 M NaOAc pH 8.0. DNA was precipitated again at -80°C with an equal volume of isopropanol. DNA was pelleted by centrifugation and resuspended in 0.3 M NaOAc pH 8.0. DNA was precipitated again at -80°C with two volumes of 95% EtOH. Pelleted DNA was washed with 70% EtOH and resuspended in 50 µl TE (Tris EDTA).

Isolation of Genomic DNA from R. rhodochrous Strain IGTS8

IGTS8 genomic DNA was isolated as described. 20 ml RM was inoculated with a single colony of IGTS8 and incubated at 30°C for 48 hours with shaking at 220 rpm. Cells were harvested by centrifugation (5000xg). Cells were resuspended in 10ml TE (10 mM Tris Base, 1 mM EDTA) with 100 mg lysozyme and incubated for 30 minutes at 30°C. Cells were lysed by adding 1 ml of 20% sodium dodecyl sulfate (SDS). 10 ml of TE-saturated phenol and 1.5 ml 5 M NaCl were added immediately and the mixture was gently agitated for 20 minutes at room temperature. Phenol was

removed by centrifugation, and the aqueous layer was extracted twice with an equal volume of chloroform. An equal volume of isopropanol was added to the aqueous layer to precipitate the DNA. DNA was spooled onto a pasteur pipette and redissolved in TE. DNA was then RNased with 20 µg/ml RNA for 1 hour at 37°C. The sample was made to a final concentration of 100 mM NaCl and 0.4% SDS and proteased with 100 µg/ml protease K. The sample was then extracted with phenol and chloroform and precipitated with isopropanol as before. The purified genomic DNA, which included the DNA of the present invention, was resuspended in TE.

Construction of Plasmid Library of IGTS8

Genomic DNA from the Dsz+ source organism (IGTS8) was cut with TaqI in order to produce fragments 0.5 - 23 kb in length. Cut DNA was electrophoresed through 0.8% low melting temperature agarose and DNA fragments greater than 5 kb in length were isolated and purified by standard methods (Maniatis, T. et al. (1982), MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Laboratory Press)). Vector pRR-6 was cut with NarI to completion. The vector ends were dephosphorylated with HK™ phosphatase to prevent religation of the vector. The size-fractioning genomic DNA was ligated to cut and dephosphorylated pRR-6.

Molecular Complementation of Dsz- Mutant Strain CPE-648

Plasmid library ligations (above) were used to transform Dsz- mutant strain CPE-648 by electroporation as described. Negative control transformations of CPE-648, which did not contain DNA (mock transformations), were also performed. After the four hour incubation in RM, the c lls were spun out of suspension by centrifugation and the supernatant was removed. The cells were resuspended in BSM with no sulfur. These cells were used to inoculate 250 ml of BSM supplemented with 300 µl of a saturated

ethanol solution of DBT. By this procedure, clones which are capable of complementing the Dsz- mutation will be selected by the sulfur bioavailability assay. Strains containing the complementing sequences (i.e., the DNA of the present invention) will successfully remove the sulfur from DBT and grow preferentially.

After 6 days incubation at 30°C, the cultures were assayed for 2-HBP by HPLC. Accumulation of 2-HBP was detected in experimental cultures while no accumulation of 2-HBP was detected in control cultures. The culture producing 2-HBP was spread onto RM plates supplemented with chloramphenicol to obtain single colonies that were harboring plasmids. These plates were replica-plated to BSM agarose plates supplemented with 1.2 ml/liter of a saturated ethanol solution of DBT. After 24 hours incubation at 30°C, 2-HBP could be detected around some individual colonies under short wave UV illumination. These colonies presumably harbored plasmids which complemented the Dsz- mutant by restoring the former Dsz+ phenotype.

Characterization of Clones Complementing Dsz- Mutant CPE-648

Two independent plasmid libraries successfully complemented mutant CPE-648 to Dsz^+ as described above. Plasmid DNA was isolated from single colonies which demonstrated 2-HBP production on BSM plus DBT plates (above) from cultures transformed with each of the two libraries. This plasmid DNA was used to transform E. coli strain JM109. Plasmid DNA was isolated and cut with restriction endonucleases in order to build a restriction map of the clones. Each of the two libraries yielded a single complementing clone. By restriction pattern similarities, the two clones appear to have overlapping sequences. These clones have been d signat d pTOXI-1 (Figure 4) and pTOXI-2, respectively. pTOXI-1 contains an insert of

approximately 6.6kb. pTOXI-2 contains an insert of approximately 16.8kb.

Complementation of Dsz⁻ Mutant GPE-362

Dsz- mutant GPE-362 was transformed with plasmids pTOXI-1 and pTOXI-2. As a control, GPE-362 was also transformed with vector pRR-6. Transformants containing plasmid DNA were selected on RM plus chloramphenicol plates. Cm^R colonies were transferred to BSM agarose plates supplemented with DBT. After 24 hr. incubation at 30°C, 2-HBP production could be seen around colonies containing either pTOXI-1 or pTOXI-2 by short wave UV illumination. No 2-HBP could be detected around colonies containing only vector pRR-6.

Overexpression of the Dsz⁺ Trait Upon Reintroduction of Cloned DNA

Plasmids pTOXI-1 and pTOXI-2 were transformed into Dsz- mutant strain CPE-648. Transformants containing plasmid DNA were selected on RM plus chloramphenicol plates. The specific activity of individual clones was examined by the following protocol.

Single colonies of CPE-648 containing either pTOXI-1 or pTOXI-2 were used to inoculate 25 ml RM plus 25 µg/ml chloramphenicol in a 250 ml flask. As a positive control, parent strain IGTS8 was also grown in 25 ml RM. After 48 hours of growth at 30°C, 225 rpm shaking, 2.5 ml of the cultures were crossed into 25 ml BSM supplemented with 0.7 mM DMSO. Cultures were incubated for an additional 40 hours at 30°C. The optical density of each culture was measured at 600 nm against an appropriate blank. DBT was added to a final concentration of 150 µM and the cultures were incubated for 3 hours at 30°C. An equal volume of Reagent Alcohol (Baxter, McGaw Park, IL) was then added to each culture to solubilize any remaining DBT or 2-HBP. A 1 ml sample was removed and cellular debris removed by

centrifugation. The supernatant was analyzed for 2-HBP by the HPLC assay described above. The specific activity is calculated as mg of 2-HBP per liter/hours of incubation/OD_{600}. The results of the above assay is listed in Table 1.

TABLE 1: Biocatalytic Desulfurization Activity of Transformed Mutants

STRAIN	OD_{600}	2-HBP (mg/l)	Specific Activity (mg/l/hr/OD_{600}
IGTS8	2.89	3.94	0.45
GPE-362	1.53	0.00	0.00
CPE-648	4.10	0.00	0.00
CPE648 (pTOXI-1)	3.84	15.84	1.37
CPE648 (pTOXI-2)	2.88	5.74	0.66

EXAMPLE 2: DNA SEQUENCING OF A DESULFURIZATION ACTIVE BIOCATALYST BY THE DIDEOXY METHOD FROM PLASMID PTOXI-1

Materials

Bacterial strains and plasmids

Plasmid pTOXI-1 was used as the original source of DNA for sequencing. Escherichia coli strain JM109 was used as a host for subcloning and plasmid maintenance. Plasmids pUC18 and pUC19 were purchased from Bethesda Research Laboratories (Bethesda, MD).

Enzymes and Reagents

Restriction endonucleases were purchased from Beth sda Research Laboratories (BRL) and New England Biolabs (Beverly, MA). T4 ligase was purchased from BRL. A

Sequenase Version 2.0 DNA sequencing kit was purchased from United States Biochemical Corporation (Cleveland, OH). All enzymes and kits were used in accordance with manufacturer's recommendations.

Growth Media and conditions

E. coli strain JM109 harboring plasmids was grown in L-broth (Difco) containing 100 µg/ml ampicillin. Transformants were selected on L-plates supplemented with 1.5% agar and containing 100 µg/ml ampicillin. E. coli strains were grown at 37°C.

Methods

Plasmid DNA preparation from E. coli

Plasmid DNA was prepared from E. coli via lysis by SDS (Maniatis, et al.). The DNA was further purified through a polyethylene glycol precipitation before use in sequencing reactions.

Plasmid Subcloning

The following subclones of pTOXI-1 were generated by standard techniques to aid in DNA sequencing:

a) pMELV-1 (Figure 5) was derived by isolating the 6.7kb HinddIII/NdeI fragment from pTOXI-1 (shown in Figure 4) and ligating it to pUC-18 cut with HindIII/NdeI. JM109 cells harboring pMELV-1 were identified by plasmid isolation and restriction endonuclease analysis (Maniatis, et al.).

b) pSMELV-1A (Figure 6) contains the 1.6kb SphI/XhoI fragment of pMELV-1 subcloned into pUC-18.

c) pSMELV-2A (Figure 6) contains the 0.7kb BamHI/SacI fragment of pMELV-1 subcloned into pUC-18.

d) pSMELV-3A (Figure 6) contains the 3.5kb SacI/XhoI fragment of pMELV-1 subcloned into pUC-18.

e) pSMELV-4A (Figure 6) contains the 1.5kb SphI/BamHI fragment of pMELV-1 subcloned into pUC-18.

Dideoxy Sequencing from Plasmid DNA

a) Denaturation. Prior to sequencing reactions, plasmid DNA must be denatured. This was accomplished by treatment with NaOH. The denatured DNA is then recovered by addition of salt and EtOH precipitation. Preferably, 2-5 µg of denatured plasmid DNA is used in each sequencing reaction. See manufacturer's recommendations with Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation).

b) Dideoxy sequencing. Chain termination dideoxy sequencing with Sequenase 2.0 was performed as described by the manufacturer (U.S. Biochemical Corporation). Sequencing of the cluster was initiated by priming subclones pMELV-1A, pMELV-2A, pMELV-3A, pMELV-4A with the "-40 Universal Primer" defined as: 5'-GTTTTCCCAGTCACGAC-3' and the "Reverse Primer" defined as: 5'-AACAGCTATGACCATG-3'. The sequence was extended by synthesizing overlapping oligonucleotides to previously read sequence using the Gene Assembler Plus (Pharmacia, Piscataway, NJ). The synthesized oligonucleotides were used as primers for continuing sequence reactions. Plasmid pMELV-1 was used as the template for all of the remaining sequences. DNA sequence was read from both strands of the plasmid clone to increase fidelity.

EXAMPLE 3: COMPLEMENTATION CLONING OF A DESULFURIZATION ACTIVE BIOCATALYST FROM A COSMID LIBRARY; TRANSFECTION OF BIOCATALYST DNA INTO AN R. FASCIANS HOST ORGANISM

Materials and Methods

Bacterial strains, media and reagents

Rhodococcus sp. Rhodococcus rhodochrous strain IGTS8, obtained from th Institute of Gas Technology (Chicago,

IL) was used. UV1 is a mutant of IGTS8 that is unable to desulfurize DBT, described herein. R. fascians D188-5 (Desomer, et al., J. Bacteriol., 170:2401-2405, 1988) and R. rhodochrous ATCC13808 (type strain from ATCC) do not metabolize DBT. E. coli XL1-Blue (from Stratagene Cloning System, La Jolla, CA) is recA1 lac thi endA1 gyrA96 hsdR17 supE44 relA1 [F' proAB lacI^q lacZΔM15 Tn10]. E. coli CS109 is W1485 thi supE F'. E. coli S17-1 is a derivative of E. coli 294 and is recA thi pro hsdR$^-$ res$^-$ mod$^+$ [RP4-2-Tc::Mu-Km::Tn7] (Simon, et al., Plasmid vectors for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria, p. 640-659. In A. Weissbach, and H. Weissbach (eds.), Methods in enzymology, vol 118, Academic Press, Inc., Orlando, 1986).

Pseudomonas minimal salts medium (PMS) was prepared according to Giurard and Snell (Biochemical factors in growth, p. 79-111. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (eds.), Manual of methods for general bacteriology, American Society for Microbiology, Washington, DC., 1981) and contained 0.2% glycerol, 40 mM phosphate buffer (pH 6.8), 2% Hutner's mineral base, and 0.1% $(NH_4)_2SO_4$. PMS medium lacking sulfate was prepared with chloride salts in place of sulfate salts. Luria broth (LB) was 1% bactotryptone, 0.5% yeast extract, and 1% NaCl. All liquid medium incubations were performed with shaking in water baths (New Brunswick Scientific, Edison, NJ). Ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml) were included as selective agents when required. Dibenzothiophene (DBT) was purchased from Fluka Chemical Corporation of Ronkonkoma, NY. DBT-sulfoxide was from ICN Biochemicals of Irvine, CA, and DBT-sulfone was obtained from Aldrich Chemical Company of Milwaukee, WI. Agarose was obtained from BRL.

Plasmid vectors

pLAFR5 (Keen, et al., Gene 70:191-197, 1988) and pRF29 (Desomer, et al., 1988) served as sources of the Rhodococcus plasmid origin of replication.

Cosmid library construction

High molecular weight DNA was isolated from IGTS8 by the method of Consevage et al, (J. Bacteriol., 162:138-146, 1985), except that cell lysis was accomplished in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing lysozyme (5 mg/ml) and SDS (2%). The DNA was partially digested with Sau3AI and fragments of approximately 20 kb were isolated after centrifugation through a sodium chloride gradient (Frischauf, et al., Digestion of DNA: size fractionation, p. 183-189. In S. L. Berge, and A. R. Kimmel (eds.), Methods in Enzymology, vol 152, Academic Press, Inc, San Diego, CA, 1987). These fragments were ligated into the BamHI site of pLAFR5 using standard procedures. In vitro packaging was performed using Gigapack Plus (Stratagene). Packaged cosmids were transduced into E. coli S17-1.

DBT spray plate assay

A spray plate assay for the identification of bacteria capable of modifying dibenzothiophene (DBT) was originally described by Kiyohara et al, (Appl. Environ. Microbiol., 43:454-457, 1982) and modified by Krawiec (Bacterial desulfurization of thiophenes: screening techniques and some speculations regarding the biochemical and genetic bases, p. 103-114. In G. E. Pierce (ed.), Developments in Industrial Microbiology, vol 31, Society for Industrial Microbiology, Columbus, Ohio, 1990). The assay was further modified for use with R. rhodochrous IGTS8 as follows. Cells from individual IGTS8 colonies were transferred to LB plates as small (0.5 cm) patches and were incubated at 30°C for 24 to 36 h. Large amounts of cells from these patches were transferred onto PMS-1% agarose

plates that lacked a source of sulfur. These plates were immediately sprayed with a 0.1% DBT solution in ethyl ether. The PMS-DBT plates were incubated at 30°C for a minimum of 18 hours and fluorescent products around the patches were detected by viewing under short-wave (254 nm) UV illumination.

Sulfur bioavailability assay

IGTS8 was incubated in PMS medium at 30°C for 24 to 48 h, the cells were pelleted by centrifugation, followed by two washes with sulfur-free PMS. Washed cells were inoculated into PMS that contained, as a sole source of sulfur, a 0.2% concentration of one of the following: DBT, DBT-sulfoxide, or DBT-sulfone. The inoculum was adjusted so that the beginning absorbance at 600 nm (A_{600}) was 0.02. The culture was incubated at 30°C and growth was monitored at A_{600}. For cultures incubated with DBT, the supernatant was viewed at various intervals under short wave UV light to check for production of fluorescent products.

Plasmid isolation and hybridizations

Cosmid DNA (pLAFR5) was isolated from E. coli as described by Ish-Horowicz and Burke (Nucl. Acids Res., 9:2989-2998, 1981), and from Rhodococcus species as described by Singer and Finnerty (J. Bacteriol., 170:638-645, 1988). Large scale cosmid preparations were carried out according to Birnboim and Doly (Nucl. Acids Res., 7:1413-1423, 1979). DNA hybridization experiments were performed according to Southern (J. Molec. Biol., 98:503-517, 1975). DNA was labelled with ^{32}P-dCTP (Amersham), using the random primer method of Feinberg and Vogelstein (Anal. Biochem., 137:266-267, 1984).

UV mutagenesis of IGTS8

IGTS8 was incubated overnight in LB at 30°C and approximately 3000 colony forming units were spread onto

fresh LB plates. These plates were immediately exposed to short wave UV light (254 nm) for 5 to 20 s at a distance of 3.5 cm. Plates were incubated at 30°C for 48 h or until colonies developed. Colonies from plates exhibiting >50% cell death were assayed for their ability to metabolize or desulfurize DBT, using the spray plate assay.

Electrotransformation of Rhodococcus

R. rhodochrous IGTS8 and the UV1 mutant were transformed with plasmid DNA via electroporation (Gene Pulser, Biorad Laboratories, Inc, Hercules, CA). The bacteria were grown in LB for 24 to 48 h at 30°C, diluted to an A_{600} of 0.15 with fresh LB, and incubated at 30°C for an additional 4 h. Cells were collected by centrifugation and washed four to five times with 0.3 M sucrose and finally resuspended to ~5 x 10^9 cells/ml in 0.5 M sucrose. To an ice cold 0.2 cm electroporation cuvette (Biorad), was added 40 µl of this bacterial solution. The cells were pulsed at 25 µF and 2.5 kV with the Pulse Controller at 800 ohms and were immediately diluted with 1 ml of LB containing 0.5 M sucrose. The cells were incubated at 30°C for 1 h, plated on LB agar plates plus appropriate antibiotics, and incubated at 30°C until colonies developed. When the plasmid carried the pRF29 Rhodococcus plasmid origin of replication, colonies were visible after 48 h. In the absence of the pRF29 origin, colonies appeared after 4 to 5 days.

R. fascians D188-5 was transformed by electroporation in a similar manner but, due to its slower growth rate, it was incubated in LB overnight until it reached an A_{600} of ~2.0. The cells were washed and resuspended in distilled water instead of sucrose. The Pulse Controller was set at 400 ohms and the recovery period after electroporation was in LB for 4 h before plating onto selective media. Successful transformation of R. fascians D188-5 with E. coli plasmids required that the DNA be methylated in vitro

beforehand, using the CpG methylase, SssI (New England Biolabs, Inc., Beverly, MA).

Gas chromatography and mass spectroscopy

Cells were incubated overnight in LB medium at 30°C and 100 µl was used to inoculate 50 ml of PMS minimal medium. The culture was incubated at 30°C for 4 days, washed twice with sulfur-free PMS and the pelleted cells were inoculated into 50 ml of PMS that contained 0.1% DBT as the sole source of sulfur. These cells were incubated at 30°C for 24 h and the supernatant was stored frozen at -20°C. For assays involving R. fascians D188-5, incubation times were increased 2 to 3-fold.

Sample preparation and chemical analyses were performed as described (Olson, et al., Energy & Fuels, submitted, 1993). Briefly, each sample supernatant (~50 ml) was thawed and residual insoluble material was removed by centrifugation. The cleared supernatant was acidified with HCl to pH 1.0 and then extracted three times with 50 ml of ethyl acetate. Insoluble material from the centrifugation step was also extracted with ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous calcium chloride, filtered, and ethyl acetate was removed by rotary evaporation. A known amount of internal standard (octadecane in chloroform solution) was added to the sample, which was then analyzed by GC/FID (gas chromatography/flame ionization detection) and GC/FTIR/MS (gas chromatography/Fourier transform infrared/mass spectrometry). In some samples, the acidic components in the ethyl acetate extract or in the post-extraction aqueous layer were methylated by treating with an ether solution of diazomethane.

The analyses were performed on a serially interfaced GC/FTIR/MS system as previously described (Diehl, et al., Spectros. Int. J., 8:43-72, 1990, Olson and Diehl, Anal. Chem., 59:443-448, 1987). This system consisted of the

Finnegan ion trap (ITD 800) operated with the AGC on and the Nicolet 20SXB Fourier transform infrared spectrometer. Gas chromatography was conducted with a 30 m x 0.32 mm DB5 column (1.0 µm phase thickness) with a 2.0 ml/min helium carrier flow rate measured at 330°C. On-column injections were utilized for sample introduction because the sulfoxides and sulfones are thermally unstable and they decompose in split or splitless injectors (Vignier, et al., J. High Resol. Chromatogr. & Chromatogr. Commun., 6:661-665, 1983). The oven temperature program was as follows: 40°C injection, followed by increases in temperature at rates of 20°C/min to 80°C, 5°C/min to 200°C, 10°C/min to 330°C, and hold for 5 min. GC/FID analyses were performed with a HP 5880A with a similar column and program for flow rate and oven temperature.

Results

Isolation of a Dsz- Mutant of R. rhodochrous IGTS8

When cloning from a foreign bacterial genus into E. coli, not all genes are expressed nor are all protein products active. To assure that cloned desulfurization genes would be expressed in the host cell, a mutant of R. rhodochrous IGTS8 that could no longer desulfurize DBT was isolated. Using this mutant as a cloning recipient would insure that the cellular environment was appropriate for gene expression and protein function, thereby allowing screening for cloned desulfurization genes by complementation.

R. rhodochrous IGTS8 was mutagenized by exposure to UV light, and 1000 survivors were screened for the ability to produce a UV fluorescent product in the DBT spray plate assay. Three potential desulfurization negative mutants were identified and then re-evaluated in the sulfur bioavailability assay. Two mutants (designated UV1 and UV23) could not use DBT or DBT-sulfone as sole sources of sulfur and thus appeared to be Dsz-. When gr wn in the presence

of DBT, mutant UV1 could not metabolize DBT to 2-HBP or to any other potential intermediate, as measured by GC/MS analysis. Therefore, strain UV1 was considered to be Dsz- and was used as the host for complementation studies to identify clones that carried desulfurization genes.

Cosmid cloning of desulfurization genes

DNA from Dsz+ source organism IGTS8 was used to construct a library in the cosmid vector, pLAFR5. This library was transduced into E. coli S17-1 and plasmids were isolated from approximately 25,000 colonies. These cosmids were electroporated into R. rhodochrous UV1, a Dsz- mutant of IGTS8, with an efficiency of ~300 transformants/μg DNA. Various numbers of UV1 transformants were pooled and incubated for 18 hours at 30°C, after which the cells were washed twice and resuspended in sulfate-free PMS. Approximately 7 x 10^8 pooled cells were inoculated into 100 ml of PMS with DBT as the sole source of sulfur. A predicted product of the DBT desulfurization reaction is 2-HBP, which is fluorescent when exposed to UV light. Therefore, batch cultures were grown at 30°C and the supernatants were observed for fluorescence. Approximately 3300 UV1 transformants were screened in four separate batches. In one batch (representing ~600 transformants) a UV fluorescent product appeared in the supernatant after five days' incubation. Individual colonies were isolated and twelve of these continued to produce a fluorescent product when exposed to DBT.

Attempts to recover cosmid DNA from these isolates failed, so Southern hybridizations were performed to determine if the cosmids had become integrated into the chromosome of strain UV1. Chromosomal DNA was isolated from seven transformants and digested with EcoRI. After agarose electrophoresis and blotting, the fragments were hybridized with ^{32}P-labelled probes derived from pLAFR5. In all transformants tested, pLAFR5 probes hybridized to a

DNA fragment ~20 kb in size. Vector derived probes did not hybridize to the control IGTS8 genome. Therefore, the desulfurization positive cosmid clones had apparently integrated into the chromosome of strain UV1.

Since the plasmids had integrated into the chromosome, the genomic DNA connected to either side of the plasmid cloning site must represent R. rhodochrous IGTS8 sequences that were able to complement the Dsz- mutation in strain UV1. (This would be true regardless of whether the mode of integration was by homologous or illegitimate recombination.) Sequences were recovered that flanked the inserted plasmid from three desulfurization positive transformants by digesting genomic preparations with EcoRI or BamHI. These enzymes cut pLAFR5 once in the polylinker region so that an intact sequence of pLAFR5 could be recovered, linked to a neighboring chromosomal fragment from IGTS8. The digested DNA was ligated to itself (at a concentration of ~20 ng/µl) and was transformed into E. coli S17-1. Sixteen tetracycline resistant colonies were obtained, seven from the BamHI digestion and nine from the EcoRI digestion. Restriction enzyme analysis revealed that all the EcoRI-rescued clones contained a 2.1 kb fragment of IGTS8 DNA. The BamHI-rescued clones contained a 1.65 kb fragment from IGTS8.

The 2.1 kb IGTS8 DNA from the EcoRI rescue experiment was used as a template to make labelled DNA probes, which were hybridized to colony lifts of the original, intact cosmid library in E. coli. Of 5000 colonies, 17 hybridized with the IGTS8 probes. Cosmid DNA was isolated from each clone and transformed into strain UV1. Three of the seventeen DNA preparations complemented the Dsz- phenotype.

A restriction map for this region was constructed, using EcoRI and HindIII. Probes from the 2.1 kb IGTS8 DNA hybridized to the 4.5 kb EcoRI fragment. All cosmid clones that conferred the Dsz+ phenotype contained the

entire 4.5 kb EcoRI fragment and portions of the 4.5 kb EcoRI-HindIII and 18 kb EcoRI fragments. These results indicated that the desulfurization genes lay within a 15 kb region.

Subcloning the desulfurization genes

The 4.5 kb EcoRI and the 4.5 kb EcoRI-HindIII fragments were subcloned into pLAFR5, but neither fragment complemented the Dsz- mutation of strain UV1. The 9.0 kb EcoRI fragment from GE1-H, the 15.0 kb EcoRI-HindIII fragment from GE1-C, and the 18 kb EcoRI fragment from GE1-K were subcloned into pLAFR5 to yield the plasmids pSAD60-28, pSAD48-12, and pSAD56-6, respectively. When transformed into UV1, all three produced UV fluorescent products from DBT in the spray plate assay, consistent with the localization of the Dsz+ phenotype as determined by restriction mapping. Construction of additional subclones from this region narrowed the location of the relevant genes to a 6.5 kb BstBI fragment.

Nature of the mutation in strain UV1

Genomic blots of EcoRI digested IGTS8 and UV1 DNA were hybridized with probes produced from the 2.1 kb EcoRI-rescued fragment of IGTS8. No hybridization was detected to UV1 DNA, indicating that the UV1 mutation is a large deletion and not a simple point mutation.

A Rhodococcus plasmid origin of replication increases transformation of UV1

Electroporation of UV1 with pSAD48-12 typically resulted in a low transformation efficiency (~550/µg DNA) and only about 50% of the transformants exhibited the Dsz+ phenotype (presumably because DNA had been lost or rearranged during recombination with the chromosome). To improve the transformation efficiency, a 4.5 kb HindIII fragment from pRF29 was cloned into the HindIII site of

pSAD48-12, resulting in pSAD74-12. This 4.5 kb fragment contains a Rhodococcus plasmid origin of replication, which allowed pSAD74-12 to replicate as a plasmid in strain UV1. This clone transformed UV1 with an efficiency of greater than 10^4 transformants/µg DNA. Nearly 100% of these transformants exhibited the Dsz+ phenotype. Unfortunately, the yield of plasmid prepared directly from UV1 was so poor that DNA from minipreparations could not be visualized on agarose gels. However, plasmid isolated from UV1 could be used to transform E. coli S17-1, from which large amounts of the plasmid were prepared.

The Dsz+ phenotype is not expressed in E. coli S17-1

E. coli S17-1 was transformed with pSAD48-12 and desulfurization activity was measured with the spray plate assay. No positive colonies were identified. It was possible that the E. coli polymerase could not recognize the IGTS8 promoter(s) in pSAD48-12, so the IGTS8 DNA was placed under control of the E. coli lac promoter. The 15 kb EcoRI-HindIII IGTS8 fragment from pSAD48-12 was subcloned into the pBluescript vectors, SK⁻ and KS⁻, so that the IGTS8 fragment was cloned in both orientations with respect to the lac promoter. Neither clone expressed the Dsz+ phenotype in E. coli XL1-Blue. It is not yet known whether this stems from poor transcription or translation of the cloned genes or whether the overproduced proteins are inactive in E. coli S17-1.

The Dsz+ gene or genes are expressed in R. fascians

Since the cloned genes were either not expressed or produced inactive proteins in E. coli, efforts were initiated to express the genes in other Rhodococcus species. R. fascians D188-5 exhibited no desulfurization in the DBT spray plate assay or in the sulfur bioavailability assay. Initial attempts to transform R. fascians with the desulfurization positive plasmid, pSAD74-12 were unsuccessful.

Other Rhodococcus species are known to have endogenous restriction systems that cleave DNA at SalI-like restriction sites. Since pSAD74-12 contained multiple SalI recognition sequences, CpG methylase, SssI, was used to methylate pSAD74-12 in vitro. With methylated pSAD74-12 DNA, transformants of R. fascians D188-5 were obtained with an efficiency of about 7 x 10^3 transformants/μg DNA. These transformants displayed the Dsz+ phenotype in the spray plate assay and GC analysis of liquid medium supernatant revealed the formation of 2-HBP from DBT.

Efforts to transform pSAD74-12 into a second species, R. rhodochrous ATCC13808 were ineffective, despite the use of unmethylated or CpG-methylated plasmid. It is possible that the electroporation conditions for ATCC13808 were not optimal, though a wide range of conditions was tested. It seems more likely that ATCC13808 has a restriction system that is not inhibited by CpG methylation.

2-HBP is the major desulfurization product

The predominant metabolite produced from DBT by R. rhodochrous IGTS8 is 2-HBP, with small amounts of 2'-hydroxybiphenyl-2-sulfinic acid (DBT-sultine) and 2'-hydroxybiphenyl-2-sulfonic acid (DBT-sultone) also identified by GC/MS analysis (Olson, et al., Energy & Fuels in press, 1993). These products were also produced by IGTS8 in this work (Table 2). Neither R. fascians D188-5 nor R. rhodochrous Dsz- mutant UV1 produced these products from DBT. However, when R. fascians D188-5 was transformed with plasmid pSAD74-12 and when the R. rhodochrous UV1 mutant was transformed with plasmid pSAD104-10, these bacteria produced products from DBT that were identical to those identified for R. rhodochrous IGTS8 (Table 2). In particular, 2-HBP was produced in large quantities, indicating that carbon-sulfur bond specific desulfurization of DBT was mediated by products of genes cloned from IGTS8.

One subclone, pSAD90-11, carried a DNA fragment that was supposedly identical to that cloned into pSAD104-10, but the two plasmids differed in the results they produced when introduced into R. rhodococcus UV1. In the plate assay, the surface film of DBT disappeared from the vicinity of colonies that contained pSAD104-10, producing a clear zone, and a fluorescent halo appeared around those colonies. On the other hand, when cells contained pSAD90-11, no fluorescent products were produced but a zone of DBT clearing did form around each colony. GC/MS analysis showed that no 2-HBP was produced by cells containing pSAD90-11, but that a significant amount of DBT-sultone did accumulate (Table 2). The sultone does not accumulate in the parent strain, UV1 (data not shown). These observations imply that when the 9.0 kb EcoRI fragment was subcloned into pSAD90-11 the DNA was damaged so as to inactivate the gene(s) encoding the enzyme(s) that convert the sultone to 2-HBP. This suggests that at least two enzymes are involved in desulfurization and that the sultone may be an intermediate in the pathway. This result is consistent with the kinds of metabolites detected in the original isolate, R. rhodochrous IGTS8 (Olson, et al., 1993).

Table 2

Metabolites produced from DBT by Rhodococcus species transformed with subclones derived from R. rhodochrous IGTS8.

Metabolite[a]	Rhodococcus species (plasmid)			
	R. rhodochrous IGTS8	UV1 (pSAD104-10)[b]	UV1 (pSAD90-11)[c]	R. fascians D188-5 (pSAD-74-12)[d]
DBT	++++[e]	++++[e]	++++	++
DBTO	+	0	0	0
$DBTO_2$	0	0	0	0
DBT-sultone	+	++	++	+
DBT-sultine	0 or trace	0	trace	+
2-HBP	+++++	+++++	0	+++

a Products are: DBT, dibenzothiophene; DBTO, dibenzothiophene 5-oxide (sulfoxide); $DBTO_2$, dibenzothiophene 5,5-dioxide (sulfone); DBT-sultone, 2'-hydroxybi-

phenyl-2-sulfonic acid (detected as dibenz[c,e][1,2]-oxathiin 6,6-dioxide); DBT-sultine, 2'-hydroxybiphenyl-2-sulfinic acid (detected as dibenz[c,e][1,2]-oxathiin 6-oxide); dibenzothiophene sulfone; 2-HBP, 2-hydroxybiphenyl (Krawiec, pg. 103-114. In G. E. Pierce (ed.), Developments in Industrial Microbiology, vol 31, Society for Industrial Microbiology, Columbus, Ohio, 1990).

b 9.0 kb EcoRI DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.

c Mutated 9.0 kb EcoRI DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.

d 15.0 kb EcoRI-HindIII DNA fragment from IGTS8 subcloned into pLAFR5, plus the origin of replication from pRF29.

e Presence of metabolites is reported in relative amounts from very large amounts (+++++) to very small (+), i.e., trace amounts.

IGTS8 cannot use DBT-sulfoxide as a sulfur source

R. rhodochrous IGTS8 was incubated in minimal medium with one of the following as the sole source of sulfur:

DBT, DBT-sulfoxide, or DBT-sulfone. IGTS8 was incapable of utilizing the sulfur supplied by DBT-sulfoxide but grew well in the presence of DBT or DBT-sulfone. DBT-sulfoxide was not toxic to cells when grown in a rich medium (LB). Therefore, either IGTS8 cannot transport or otherwise act on DBT-sulfoxide, or else DBT-sulfoxide is not a true intermediate of the desulfurization pathway.

EXAMPLE 4: DNA SEQUENCING OF A 9763 NUCLEOTIDE ECORI-SAU3AI FRAGMENT CONTAINING THE GENE OR GENES FOR THE DESULFURIZATION BIOCATALYST OF IGTS8 BY THE METHOD OF SANGER ET AL.

A 9763 nucleotide EcoRI-Sau3AI fragment containing the gene or genes responsible for the Dsz+ phenotype was isolated from the IGTS8 source organism. The DNA sequence of this fragment was determined from both strands of DNA using the dideoxy chain-termination method of Sanger et al. (1977), DNA sequencing with chain-termination inhibitors, 74 PROC. NATL. ACAD. SCI. USA 5463-5467, a modified T7 DNA polymerase (USB) and [α-^{35}S]-dCTP (Amersham). Deletion clones for DNA sequencing were constructed in pBluescript (Stratagene) using exonuclease III and the methods of Henikoff (1984), Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing, 28 GENE 351-359.

Sequences from 141 individual deletion clones were used to reconstruct the entire 9763 nucleotide fragment.

Computerized sequence assembly was performed using DNA InspectorII (Textco, Hanover, NH). The DNA sequence was determined independently for each strand of DNA, but the entire 9763 nucleotide fragment was not completely sequenced on both strands. The sequence determined from one strand of DNA covered 95% of the 9763 nucleotide sequence. On the other DNA strand, 96% of the sequence was determined. The sequence was determined from at least two independent deletion clones for the entire 9763 nucleotide fragment.

EXAMPLE 5: FURTHER RESOLUTION OF THE SEQUENCE OF PTOXI-1 AND OPEN READING FRAMES (ORFS) ENCODED THEREIN; DSZ+ PROMOTER ENGINEERING; EXPRESSION OF THE DSZ+ PHENOTYPE IN A HETEROLOGOUS HOST ORGANISM; MAXICELL ANALYSIS OF DESULFURIZATION GENE EXPRESSION PRODUCTS

Organization of the desulfurization cluster

Sequencing of pTOXI-1, the results of which are set forth below in the Sequence Listing, predicted three nearly contiguous open reading frames (ORFs) on one strand of the clone (Figure 7). The sizes of each ORF are predicated as 1359 bases (bps 786-2144) for ORF 1, 1095 bases (bps 2144-3238) for ORF 2 and 1251 bases (bps 3252-4502) for ORF 3. Subclone analysis described below has revealed that ORFs 1, 2 and 3 are required for the conversion of DBT to 2-HBP and that all of the genes encoded by these ORFs are transcribed

on a single transcript as an operon. All subclones described below are maintained in E. coli - Rhodococcus shuttle vector pRR-6. Activity of each subclone was determined by growing transformants of Dsz- strain CPE-648 in a rich media (RM) for 48 hours. 1 ml of the culture was used to inoculate 25 ml BSM supplemented with greater than 100 μM DBT or DBT-sulfone. Cultures were assayed for desulfurization products after 48 - 120 hours. A diagram of each of the subcloned fragments is shown in Figure 8.

In subsequent studies, the subclones were grown in rich media with chloramphenicol, then crossed into BSM supplemented with 100 μM of either DBT or DBT-sulfone. Cultures were shaken at 30°C for 2-5 days and assayed for desulfurization products by HPLC.

A. pENOK-1: A subclone was constructed which contains the 4.0 kb SphI fragment of pTOXI-1. This fragment spans ORFs 1 and 2 but truncates ORF 3. Analysis of pENOK-1 containing transformants revealed the production of no products when incubated with DBT. However these transformants were capable of producing 2-HBP from DBT-sulfone.

B. pENOK-2: A suclone which contains the 3.6 kb SacI fragment of pTOXI-1 was constructed. This fragment contains ORFs 2 and 3 but truncates ORF 1. Analysis of pENOK-2 transformants revealed no production of any desulfurization products from either DBT or DBT-sulfone. The lack

of any activity detectable from either ORFs 2 or 3 suggests that the ORFs are arranged as an operon with transcription mediated from a single upstream promoter. Presumable, this promoter has been removed in this subclone.

C. pENOK-3: A 1.1 kb XhoI deletion mutation of pTOXI-1 was constructed. Both ORFs 1 and 2 are truncated. ORF 3 remains intact. Transformants harboring pENOK-3 show production of DBT-sulfone from DBT. No production of 2-HBP is detected from either DBT or DBT-sulfone. It should also be noted that at the nucleotide level, a deletion of this type would not result in a polar mutation. The sequence predicts an in-frame splicing of ORFs 1 and 2 which would produce a hybrid protein that is presumably inactive. However, by avoiding stop codons, the putative single mRNA transcript remains protected by ribosomes allowing for translation of ORF 3. The ability of the ORF-3 product to produce DBT-sulfone from DBT demonstrates that DBT-sulfone is a true intermediate in the carbon-sulfur bond specific biocatalytic desulfurization pathway of IGTS8.

D. pENOK-11: The 3.4 kb NcoI fragment from pTOXI-1 was subcloned into a unique NcoI site of pRR-6. This fragment contains all of ORFs 2 and 3 but truncates the 5' end of ORF1. Transformants with pENOK-11 demonstrated no desulfurizing-specific enzymatic activity towards DBT or DBT-sulfone. This indicates essential coding regions bordering

this fragment. This is consistent with the predication that the entire cluster is expressed on a single transcript as discussed for subclone pENOK-2. Again, the promoter for gene transcription is not present in this subclone. Subclone pENOK-13 (below) corroborates this prediction.

E. pENOK-13: A subclone of pTOXI-1 was constructed which had a 2.6 kb SphI-XhoI deletion. This subclone only contains an intact ORF 3. ORF 1 is lost completely and ORF 2 is truncated. This subclone showed no desulfurizing-specific enzymatic activity towards DBT or DBT-sulfone. This result should be compared with the phenotype of pENOK-3 which demonstrated production of DBT-sulfone from DBT. Because pENOK-13 differs from pENOK-3 by the additional deletion of the smaller SphI/XhoI fragment, this would indicate an element in the 1.6 kb SphI/XhoI fragment which is essential for gene expression. Because sequencing has revealed no significant ORF's contained in this region, it is postulated that a promoter element may be present in this region.

F. pENOK-16: A subclone of pTOXI-1 was designed which eliminates nearly all unnecessary sequences from the desulfurization cluster. This construct contains the 4 kb BstBI-SnaBI which presumably contains all essential sequence for complete desulfurization in that in contains all of ORFs 1, 2 and 3 as well as 234 bases of upstream se-

quence. The 3' SnaBI site lies 80 base pairs beyond the termination of ORF 3. CPE-648 harboring this plasmid was capable of converting DBT and DBT-sulfone to 2-HBP. pENOK-16 thus represents the smallest amount of the cluster yet observed which demonstrates the complete desulfurization phenotype.

G. pENOK-18: This subclone contains a NsiI-BfaI fragment of pTOXI-1. The NsiI site is 23 bp downstream of the predicted start site of ORF 1. CPE-648 harboring this subclone lacks desulfurization activity on both DBT and DBT-sulfone. This subclone most likely eliminates the promoter region and truncates the first structural gene.

H. pENOK-Nsi: To help further elucidate the start site of ORF 1, a subclone was made in which a 4 bp deletion is introduced at the unique NsiI site which is 23 bp downstream of the predicted start site of ORF1. The mutation was generated by cutting with NsiI and blunting the ends with T4 DNA Polymerase. If the NsiI site is within the first structural gene this frameshift mutation would cause an early stop signal in ORF 1. Transformants of pENOK-Nsi were capable of producing DBT-sulfone from DBT. However, no production of 2-HBP was detected indicating that the mutation had disrupted an essential structural g ne.

In subsequ nt studies, due to the clear expression of the ORF-3 encoded oxidase, in this clone, it was considered

likely that the ORF-2 product would also be expressed. Accordingly, ORF-2 alone is incapable of further metabolism of DBT-sulfone.

I. pENOK-19: A subclone of pTOXI-1 was constructed which contains a deletion from the NotI site, which is in the earlier part of ORF 2, to the SnaBI which is after ORF 3. This subclone should demonstrate the activity of ORF 1 alone. CPE648 transformants harboring this subclone displayed no enzymatic activity towards DBT or DBT-sulfone.

The results of pENOK-Nsi and pENOK-19, taken together, suggest that the ORF-I and ORF-2 products must be simultaneously expressed in order to further metabolize DBT-sulfone.

J. pENOK-20: In order to evaluate the function of ORFs 2 and 3 separately from ORF 1, DNA spanning ORFs 2 and 3 was amplified by the Polymerase Chain Reaction (PCR). Primers RAP-1 (5'-GCGAATTCCGCACCGAGTACC-3', bps 2062-2082) and RAP-2 (5'-ATCCATATGCGCACTACGAATCC-3' bps 4908-4886) were synthesized with the Applied Biosystems 392 DNA/RNA Synthesizer. Nucleotides in bold were altered from the template sequence in order to create restriction sites for subcloning; thus primer RAP-1 contains an EcoRI site, and primer RAP-2 contains an NdeI site. Amplification was carried out with the GeneAmp Kit (Perkin Elmer Cetus) which utilizes

the Taq polymerase and the Perkin Elmer Cetus 9600 Thermocycler. Parameters were as follows:

Template: pMELV-1 Plasmid DNA			0.2 or 2.0 ng
Primers:	RAP-1		0.5 or 0.2 µM
	RAP-2		0.5 or 0.2 µM
Cycles:	1X@	96°C	2 min
	25X@	96°C	30 sec
		52°C	30 sec
		72°C	2 min

Amplification yielded the predicted 2846 bp fragment. In order to express the amplified fragment harboring ORFs 2 and 3, it was ligated to the XbaI/EcoRI fragment of the chloramphenicol resistance gene promoter from Rhodococcus fascians (Desomer et al.: Molecular Microbiology (1992) 6 (16), 2377-2385) to give plasmid pOTTO-1. Ultimately, a blunt end ligation was used for the subcloning of the amplified product due to the fact that ligation using the engineered restriction sites was unsuccessful. This fusion was ligated to shuttle-vector pRR-6 to produce plasmid pENOK-20. CPE648 transformants of pENOK-20 were grown in the presence of DBT and 25 µg/ml chloramphenicol for promoter induction. All transformants converted DBT to DBT-sulfone presumably through the activity of the ORF 3 as demonstrated in subclone pENOK-3. The inability to further process DBT-sulfone with the presence of ORF 2 suggests that the product of ORF 2 alone is incapable of using DBT-sulfone as a substrate. This is consistant with results

obtained from pENOK-Nsi, and suggests that ORF-2 alone is incapable of using DBT-sulfone as a substrate.

Assignment of Gene Products of ORFs 1, 2 and 3

Based on the foregoing subclone analyses, functions have been tentatively assigned to each of the ORFs present within the pTOXI-1 sequence. ORF 3 can be identified as responsible for an oxidase capable of conversion of DBT to DBT-sulfone. Subclone pENOK-3 demonstrates this activity very clearly. ORFs 1 and 2 appear to be responsible for conversion of DBT-sulfone to 2-HBP. This aryl sulfatase activity is evidenced in subclone pENOK-1. However subclones pENOK-19 and pENOK-20 indicate that neither ORF 1 or ORF 2 alone is capable of any conversion of the intermediate DBT-sulfone. This suggests that the protein products of ORFs 1 and 2 work together to cleave both of the carbon-sulfur bonds. Presumably, this is achieved through a heterodimer arrangement of the proteins, or through a regulatory function of one protein on the other. The results of paralell investigations, presented in Example 3, suggested that ORF-1 encodes an enzyme that converts DBT--sulfone to DBT-sultone. Lengthy incubations of CPE-648 harboring pENOK-19 (intact native promoter and ORF-I) have shown neither the depletion of DBT-sulfone nor the production of any new products. This is contrary to indications derived from Example 3.

Alternative Promoter Screening

Increasing the specific activity of desulfurization is a significant objective of the studies described herein. One approach to accomplishing this goal is to replace the original promoter with one that can produce both higher and constitutive expression of the desulfurization gene cluster. Because there are so few reported and characterized Rhodococcus promoters, random genomic libraries have been prepared and screened for promoter activity in two systems. In one, the reporter is the chloramphenicol resistance gene used in the above-discussed plasmid constructions. In the other, the desulfurization cluster itself is used as a reporter.

Promoter Screening Example A. Chloramphenicol Resistance Reporter.

As also described below, partially digested Rhodococcus genomic DNA has been cloned upstream of a promoterless chloramphenicol resistance gene. The resulting libraries were then transformed into Rhodococcus which are subjected to chlorarnphenicol selection. Four apparent promoter elements were rescued by pRHODOPRO-2, although plasmid could be isolated from only one of these, possibly due to vector instability. The stable plasmid RP2-2A has been subjected to sequence analysis. Technical problems have been observ d with restriction enzyme treatment of the NarI cloning site used in these vectors. Unfortunately,

the NarI enzyme demonstrates severe site-selectivity and does not appear to digest the vector well. New vectors have been constructed in order to alleviate this problem, although a lack of convenient and unique restriction sites slowed the progress of these studies. A recent observation on the Rhodococcus replication origin will aid in constructing a more effective promoter probe, as discussed below.

Recently, the 1.4kb BglIIfragment was removed from pRR-6, and the ends were blunted and religated to produce pRR-12 (Figure 9), which contains no BglII sites. Desomer et al. (Molecular Microbiology (1992) 6 (16), 2377-2385) reported that this region was needed for plasmid replication. Thus, it was surprising that this construct was capable of producing Cm^r transformants, indicating that this region was not essential for plasmid replication in the strain of organisms used for the present studies. This observation forms the conceptual basis for construction of a vector that will utilize a synthesized BglII site for cloning the random genomic fragments. BglII accepts DNA digested by Sau3A, an effective and frequent cutter of IGTS8 DNA. These constructs are expected to allow for the production of better, more representative random genomic libraries.

Promoter Screening Example B: Desulfurization Cluster Reporter.

Vector pKAMI has been used as a second direct "shot--gun" approach to finding a suitable alternative promoter (Figure 10). An NdeI site was engineered upstream of the promoterless Dsz cluster to serve as the site of insertion of random genomic DNA (from strains GPE-362, CPE-648 and IGTS8) fractionated by NdeI and the compatible 4bp cutters MseI and BfaI. Originally, this ligation mixture was directly transformed into GPE-362 cells, which were then used en masse to inoculate 250 ml BSM + DBT. These efforts were undertaken with the goal of amplifying a superior Dsz+ strain due to its ability to utilize DBT as the sole source of sulfur. To date, 14 transformations of this type have been done. Of these, all but 2 have resulted in producing Dsz+ cultures. Eleven individual clones have been isolated and characterized. These are capable of low-level (0.6 - 1.0 mg/L 2-HBP/OD_{600}/hr), constitutive expression of the desulfurization trait. Restriction analysis of plasmids isolated from these eleven has revealed that all but one (KB4-3) are simple rearrangements of the pKAMI backbone resulting in gratuitous expression from vector borne promoters. Many of the rescued plasmids show identical restriction patterns although originating from separate ligations, suggesting an inherent vector instability. It appears as if, with this type of selection, rearrangements

of pKAMI that utilize a vector promoter sequence are strongly selected.

The above-described selection procedure has thus given way to a promoter screen geared to minimize the plasmid rearrangement. In this procedure, the pKAMI/genomic library is first amplified in E. coli, then the individual JM109 colonies are pooled together. The plasmids are extracted, and used to transform Dsz- strain GPE-362. Instead of using en masse enrichment, the GPE362 transformations are plated to Rich Media + chloramphenicol for selection of plasmid containing cells. Resulting colonies are replica-plated to BSM agarose + DBT plates, then checked for desulfurizatton activity by UV fluorescence production. Over 7,000 GPE-362 transformants have been screened in this fashion. Thirty-six have been isolated from these which produce UV fluorescence on BSM + DBT plates. Current efforts focus on the identification and characterization of the engineered plasmids borne by these 36 transformants.

Alternative Promotor Engineering

The close physical arrangement of the three ORFs of pTOXI-1 does not provide sufficient space for promoters for either ORFs 2 or 3. This fact, coupled with the results of the subclone analysis in which intact ORFs 2 and 3 provided no activity (see pENOK-2, pENOK-11, and pENOK-13), suggested that this cluster of genes is organized as an operon

with only one promoter for expression of the three genes. Given that the desulfurization trait of IGTS8 is repressed by sulfate (Kilbane and Bielaga, Final Report D.O.E. Contract No. DE-AC22-88PC8891 (1991), it is possible that the operon promoter is tightly controlled by sulfur levels. With the elucidation of the molecular arrangement of the desulfurization cluster, alternative promoters can be rationally engineered to eliminate the sulfur repression, increase expression of the desulfurization genes and thereby increase the specific activity of the Dsz^+ trait.

Examples of potential alternative promoters include other known and described promoters such as the chloramphenicol resistance gene promoter from Rhodococcus fascians (Desomer et al.: Molecular Microbiology (1992) 6 (16), 2377-2385), the nitrile hydratase gene promoter from Rhodococcus rhodochrous (Kobayashi, et al.: Biochimica et Biophysica Acta, 1129 (1991) 23-33), or other strong promoters isolated from Rhodococcus sp. by "shot-gun" promoter probing. Other potential alternative promoters include those from other Gram positive organisms such as Corynebacterium, Bacillus, Streptomyces, and the like.

Promoter Engineering Example A: Expression from the chloramphenicol resistance gene promoter from Rhodococcus fascians.

pSBG-2 (Figure 11). The promoterless desulfurization cluster was isolated from pTOXI-1 as a 4.0 kb DraI/SnaBI

fragment and ligated to a unique blunted AflII site of pRR-6. This ligation inserted the cluster downstream of the chloramphenicol resistance gene promoter and upstream of the resistance structural gene. Thus, messenger RNA (mRNA) transcription should proceed through the Dsz gene cluster and proceed on to the resistance gene. However, original selections of transformants on chloramphenicol did not yield transformants, suggesting poor transcriptional read-through. Dsz+ transformants harboring the plasmid were selected first through sulfur bioavailability assays and secondarily on chloramphenicol plates. Unlike IGTS8, pSBG-2 transformants are capable of converting DBT to 2-HBP in BSM media supplemented with 20 mM Na_2SO_4, which demonstrates the removal of sulfate repression by promoter replacement. Specific activity of transformants was measured between 0.9 and 1.7 mg 2-HBP/l/OD_{600}/hr for a 16 hr culture in a rich media (RM) supplemented with 25 µg/ml chloramphenicol.

pSBG-3. The Rhodococcus origin of replication was removed from pSBG-2 by elimination of the 4.0 kb XbaI fragment. Without the origin, transformation is obtainable only through integration. CPE-648 transformants with this plasmid were selected on RM + chloramphenicol and replica-plated onto BSM + DBT plates. Colonies were obtained which produced 2-HBP, as detected by fluorescence after 18 hr of incubation at 30°C.

Individual expression Of each ORF

Recently, studies have been initiated to express the three ORFs separately, each engineered with an alternative promoter. These studies are expected to elucidate the following: First, any potential rate limiting steps in the desulfurizafion process will be identified and overcome. Potential polarity effects of operon expression, i.e. poorer expression of downstream ORFs 2 and 3, may be causing such rate limitations. Also, given the unresolved issue of the individual functions of ORFs 1 and 2, these studies are expected to demonstrate reconstitution of DBT-sulfone to 2-HBP conversion by the separate expression of ORFs 1 and 2.

All ORFs were isolated through PCR amplification and subsequent subcloning. A typical Shine-Dalgarno sequence and a unique cloning site for alternative promoters has been engineered upstream of each ORF. Stop codons in all reading frames have been engineered downstream of each ORF to prevent read-through. Additionally, convenient flankng restriction sites for mobilization of the promoter/ORF fusions have been added to each primer. The primers used for amplification of each ORF are listed below. In-frame stop codons are marked with an asterik (*). Sequences identical to pTOXI-I template DNA are shown in bold.

ORF1UP:

```
          XbaI
5'-GGAATTCTAGACATATGAGGAACAGACCATGACTCAACAACGACAAATGC-3'
     EcoRI     NdeI           Start
```

ORF1DOWN:

```
                              Stop XbaI
3'-GTACTGTTCGGCGCAGCTGGGGACTAAGATCTTAAGC-5'
   Stop*                       Stop   EcoRI
```

ORF2UP:

```
          BglII
5'-GGAATTCAGATCTCATATGAGGAAACAGACCATGACAAGCCGCGTCGACC-3'
     EcoRI      NdeI              Start
```

ORF2DOWN:

```
                              StopBglII
3'-CGGAGTTAGCGGTGGCTATCCTTAATCTAGACTTAAGC-5'
                        Stop*  Stop     BglII
```

ORF3UP:

```
          MseI
5'-GGAATTCTTAACATATGAGGAAACAGACCATGACACTGTCACCTGA-3'
     EcoRI      NdeI              Start
```

ORF3DOWN:

```
                          MseI
3'-GACTCCTAGACTCCGCGACTAATTCTTAAGC-5'
   Stop*   Stop    Stop   EcoRI
```

Cycling parameters were: 1 x 96°C 2.0 min
25 x 96°C 30 sec
50°C 30 sec
72°C 1.0 min

Each ORF has been successfully amplified and subcloned into pUC-19 NdeI as EcoRI fragments. Alternative promoters

will be ligated into the unique NdeI sites, and the fusions will be moved to Rhodococcus-E. coli shuttle vector pRR-6 for expression in Rhodococcus.

Heterologous Expression of the Dsz+ Trait

In order to determine whether plasmid pTOXI-1 contained all of the genetic material necessary for the Dsz+ trait, heterologous expression of pTOXI-1 was attempted in Rhodococcus fascians, a related organism which does not metabolize DBT (Dsz-) and in E. coli, a non-related organism which is also Dsz-.

A. Rhodococcus fascians (ATCC 12974), a Dsz- strain, was transformed with pTOXI-1. A single transformant demonstrated UV fluorescence on BSM + DBT plates, and further analysis by HPLC clearly indicated production of 2-HBP when DBT was provided as a substrate. Thus pTOXI-1 contains sufficient information to convert a heterologous Dsz- strain to the Dsz+ phenotype.

B. E. coli strain JM109 was also transformed with pTOXI-1 and was incubated with each of the substrates DBT and DBT-sulfone in either a minimal media (BSM) or a rich media (Luria Broth). In no case was production of 2-HBP observed by HPLC analysis. The inability of E. coli to express the desulfurization genes was not unexpected as gram positive genes are not universally expressible in E. coli without promoter replacement.

In order to replace the promoter of the desulfurization cluster, a 4.0 kb DraI/SnaBI fragment was isolated from pTOXI-1. This fragment contains all of the necessary structural genes but lacks the promoter sequences. This promoterless desulfurization cluster was ligated to E. coli expression vector pDR540 (Pharmacia, Piscataway, NJ) cut with BamHI and ends made blunt with Klenow. The construction fuses the tac promoter to the desulfurization cluster. The

tac promoter is under control of the lactose repressor and is repressed in a lacI^q host such as JM109. Expression from the tac promoter is inducible by the addition of isopropyl ß-D-thiogalactopyranoside (IPTG). Transformants of JM109 harboring pDRDsz grown in Luria Broth at 30°C demonstrate the Dsz+ phenotype when incubated with DBT and induced with IPTG. A specific activity as high as 1.69 mg 2HBP/l/OD_{600}/hr has been observed with pDRDsz. Activity is greatly diminished when transformants are grown at 37°C. The highest level of activity has been observed at 1hr post induction.

The above-described expression of the Dsz+ trait in both a related and non-related heterologous host indicates that pTOXI-1 carries all of the genetic information required for conversion of DBT to 2-HBP.

Successful expression in E. coli provided a workable system in which the proteins encoded by the desulfurization cluster could be identified and characterized. Total protein from Dsz+ cells of JMI09 (pDRDsz) was isolated and examined on denaturing acrylamide gels. No novel bands could be detected with Coomassie stain. Cellular fractionation of proteins into periplasmic, cytosolic and membrane components were also analyzed by Coomassie stained gels. Again, no novel bands were detected. Without any purification, the newly expressed proteins were apparently levels too low to easily detect and resolve from background.

Maxicell Analysis of E. coli harboring pDRDsz

Proteins encoded by genes on plasmid DNA can be specifically radiolabeled in UV-irradiated cells of E. coli (Sancar, et al. Journal of Bacteriology. 1979, p. 692-693). This technique is known as Maxicell Analysis. Briefly, a recA strain of E. coli e.g. JM109 which harbors a plasmid is grown in M9CA medium (Maniatis et al.) to a density of 2 x 10^8 cells/ml. Continuously stirred cells were then subjected to UV exposure from a Mineralight Lamp Model UVG-254 (Ultro-vilet Products, Inc., San Gabriel, CA) at a distance of 10 cm

for a fluence rate of 0.5 Joules·$m^{-2}s^{-1}$. Cells were exposed for either 60, 90 or 120 seconds. The cells were then incubated at 37°C for 16 hours after which they were then washed with M9 buffer and suspended in minimal medium lacking sulfate. After 1 hour of starvation at 37°C, [^{35}S]methionine (>1000 Ci/mmol) (NEN Research Products, Boston, MA) was added at a final concentration of 5 µCi/ml and incubation was continued for 1 hour. Cells were collected by centrifugation and proteins isolated through a boiled cell procedure (Maniatis, et al.). Proteins were separated on an acrylamide gel. After the run, the gel was dried and subjected to autoradiography for 3 days.

Maxicells of JM109 harboring vector pDR540 showed only vector marker galactokinase protein. Maxicells of JM109 harboring vector pDRDsz showed the presence of three novel protein bands of sizes which correlated well with the predicted molecular weights of the three proteins responsible for the Dsz+ trait, as predicted by open reading frame analysis (see Table 3).

Table 3

Open Reading Frame	Predicted Size (kDa)	Measured Size (kDa)
ORF-1	49.5	49.5
ORF-2	38.9	33.0
ORF-3	45.1	45.0

Data obtained from Maxicell analysis thus indicated that the three predicted open reading frames of pTOXI-1 encode three structural genes which constitute the desulfurization phenotype.

The relative intensity of the three novel bands is reflective of both the number of methionine residues and the level of translation for each of the proteins. Clearly, ORF-2 with only 1 Met gives th faintest band. In

addition to the incorporation of only a single Met residue, E. coli may process the single terminal methionine, further reducing the amount of labelled protein. Therefore, the low intensity of the ORF-2 band most likely does not strictly suggest a low level of protein translation.

Interestingly, the ORF furthest from the promoter (ORF-3) appears to be present at levels comparable to ORF-1, indicating no polar effects in this operon when expressed in E. coli. It is expected that more significant information regarding protein levels will be obtained from a similar Maxicell analysis of a Rhodococcus sp. host containing plasmid pTOXI-I. Additionally, the presence of an ORF-I/ORF-2 heterodimer, postulated above, may be observable under non-denaturing conditions.

As required by 37 C.F.R. Section 1.821(f), Applicant's Attorney hereby states that the content of the "Sequence Listing" in this specification in paper form and the content of the computer-readable form (diskette) of the "Sequence Listing" are the same.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Energy BioSystems Corporation
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(ii) TITLE OF INVENTION: Recombinant DNA Encoding A Desulfurization Biocatalyst

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 02173

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 22,592
(C) REFERENCE/DOCKET NUMBER: EBC92-03A

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 790..2151

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3256..4506

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCATGCACGT CGCGCCGACG CATTTGCGCG CACGGCTCCG GGCAGTTCTC GCGGCGCTGG      60

AGGCACGGAT GGGCACCCTC AACGAACTCA CCCAAACCAC GCCGATAGCG ATCCTCGCCG     120

AAACCCTCGG CTACAGCCCT CAGACATTGG AAGCTCATGC GCGACGCATC CGGATCGACC     180

TTTGCACGCT ACGTGGCGAC GCGGCTGGAC TGACGCTGGA GGTCCGACCC GACGTGTGTG     240

GTGTAGCGCC GCTTAACGGG TGCGCACGGC GGGACATCGG CCAGCTGGCT TGCCCCTCCT     300

CCGCAGGTAG TCGACCACCC CTTCCCGCAG CGGTCGGAGG TGATCGACCG TTAGGGTCAT     360

TTGCTCGCAG ATCGGCTGAT GTTGCCGATC GACGTGGTCG ACGGGACACG CTCGCGATTG     420

GCATGGCGTC CGGTGCATAC ACGACGATCT AACCAGATCG ACGGTTTTGA GCGTCGGTCA     480

ACGTCGACTC GATGCGCCGT GCGAGTGAGA TCCTTTGTGG TGCTTGGCTA TTGACCTCGA     540

CAAGGATAGA GATTCGAAGG ACCTCGGATC GACCCAAATG CGGACGGCCG GCAGCGGCGA     600

AGGCGGCCAA GTCATCGGCA CCGTCACCGT CACCTTGACC CGACGTGCCC CGTGGTTCAA     660

GGCCTGAATT TGGCTGGTGG AGCATTGAAA TCAGGTGAAG TTTAACGGTG GGCACACCCC     720

GGGGGTGGGG GTGAGACTGC TTAGCGACAG GAATCTAGCC ATGATTGACA TTTAAAGGAC     780

GCATACGCG ATG ACT CAA CAA CGA CAA ATG CAT CTG GCC GGT TTC TTC          828
          Met Thr Gln Gln Arg Gln Met His Leu Ala Gly Phe Phe
            1               5                  10

TCG GCC GGC AAT GTG ACT CAT GCA CAT GGG GCG TGG CGG CAC ACG GAC       876
Ser Ala Gly Asn Val Thr His Ala His Gly Ala Trp Arg His Thr Asp
     15                  20                  25

GCG TCG AAT GAC TTT CTG TCG GGG AAG TAC TAC CAA CAC ATC GCC CGT       924
Ala Ser Asn Asp Phe Leu Ser Gly Lys Tyr Tyr Gln His Ile Ala Arg
 30                  35                  40                  45

ACT CTG GAG CGC GGC AAG TTC GAT CTG TTG TTT CTG CCT GAC GGG TTG       972
Thr Leu Glu Arg Gly Lys Phe Asp Leu Leu Phe Leu Pro Asp Gly Leu
                 50                  55                  60

GCC GTC GAG GAC AGC TAC GGG GAC AAC CTG GAC ACC GGT GTC GGC CTG      1020
Ala Val Glu Asp Ser Tyr Gly Asp Asn Leu Asp Thr Gly Val Gly Leu
            65                  70                  75

GGC GGG CAG GGT GCA GTC GCC TTG GAG CCG GCC AGT GTG GTC GCA ACC      1068
Gly Gly Gln Gly Ala Val Ala Leu Glu Pro Ala Ser Val Val Ala Thr
        80                  85                  90

ATG GCC GCG GTG ACC GAG CAC CTG GGT CTT GGG GCA ACC ATT TCG GCG      1116
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Met Ala Ala Val Thr Glu His Leu Gly Leu Gly Ala Thr Ile Ser Ala
 95                 100                 105

ACC TAC TAT CCC CCG TAT CAC GTT GCT CGG GTG TTC GCG ACG CTC GAT      1164
Thr Tyr Tyr Pro Pro Tyr His Val Ala Arg Val Phe Ala Thr Leu Asp
110                 115                 120                 125

CAG TTG TCA GGG GGT CGG GTG TCC TGG AAC GTC GTC ACC TCG CTC AAC      1212
Gln Leu Ser Gly Gly Arg Val Ser Trp Asn Val Val Thr Ser Leu Asn
                130                 135                 140

GAC GCT GAA GCG CGC AAC TTC GGC ATT AAT CAG CAT CTG GAA CAC GAC      1260
Asp Ala Glu Ala Arg Asn Phe Gly Ile Asn Gln His Leu Glu His Asp
            145                 150                 155

GCC CGC TAT GAC CGC GCC GAT GAG TTC TTG GAA GCG GTC AAG AAA CTC      1308
Ala Arg Tyr Asp Arg Ala Asp Glu Phe Leu Glu Ala Val Lys Lys Leu
        160                 165                 170

TGG AAC AGC TGG GAC GAG GAC GCC CTC GTG CTG GAC AAG GCG GCC GGC      1356
Trp Asn Ser Trp Asp Glu Asp Ala Leu Val Leu Asp Lys Ala Ala Gly
    175                 180                 185

GTG TTC GCC GAT CCC GCG AAG GTG CAC TAC GTC GAT CAC CAC GGG GAG      1404
Val Phe Ala Asp Pro Ala Lys Val His Tyr Val Asp His His Gly Glu
190                 195                 200                 205

TGG CTG AAT GTG CGC GGA CCT CTG CAG GTA CCG CGT TCA CCT CAG GGT      1452
Trp Leu Asn Val Arg Gly Pro Leu Gln Val Pro Arg Ser Pro Gln Gly
                210                 215                 220

GAG CCG GTG ATC CTG CAG GCC GGC CTG TCG CCC CGG GGT CGG CGC TTC      1500
Glu Pro Val Ile Leu Gln Ala Gly Leu Ser Pro Arg Gly Arg Arg Phe
            225                 230                 235

GCC GGG AAG TGG GCC GAG GCC GTC TTC AGT CTT GCA CCC AAC CTC GAG      1548
Ala Gly Lys Trp Ala Glu Ala Val Phe Ser Leu Ala Pro Asn Leu Glu
        240                 245                 250

GTG ATG CAG GCC ACC TAC CAG GGC ATC AAA GCC GAG GTC GAC GCT GCG      1596
Val Met Gln Ala Thr Tyr Gln Gly Ile Lys Ala Glu Val Asp Ala Ala
    255                 260                 265

GGG CGC GAT CCC GAT CAG ACG AAA ATC TTC ACC GCC GTG ATG CCG GTA      1644
Gly Arg Asp Pro Asp Gln Thr Lys Ile Phe Thr Ala Val Met Pro Val
270                 275                 280                 285

CTC GGC GAA AGC CAG GCG GTG GCA CAG GAA CGA CTG GAA TAT CTC AAC      1692
Leu Gly Glu Ser Gln Ala Val Ala Gln Glu Arg Leu Glu Tyr Leu Asn
                290                 295                 300

AGT CTG GTC CAT CCG GAA GTG GGA CTG TCG ACG CTA TCC AGT CAC ACC      1740
Ser Leu Val His Pro Glu Val Gly Leu Ser Thr Leu Ser Ser His Thr
            305                 310                 315

GGC ATC AAC CTG GCG GCG TAC CCT CTC GAC ACT CCG ATC AAG GAC ATC      1788
Gly Ile Asn Leu Ala Ala Tyr Pro Leu Asp Thr Pro Ile Lys Asp Ile
        320                 325                 330

CTG CGG GAT CTG CAG GAT CGG AAT GTC CCG ACG CAA CTG CAC ATG TTC      1836
Leu Arg Asp Leu Gln Asp Arg Asn Val Pro Thr Gln Leu His Met Phe
    335                 340                 345
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GCC GCC GCA ACG CAC AGC GAA GAG CTC ACG CTG GCG GAA ATG GGT CGG      1884
Ala Ala Ala Thr His Ser Glu Glu Leu Thr Leu Ala Glu Met Gly Arg
350                 355                 360                 365

CGC TAT GGA ACC AAC GTG GGG TTC GTT CCT CAG TGG GCC GGT ACC GGG      1932
Arg Tyr Gly Thr Asn Val Gly Phe Val Pro Gln Trp Ala Gly Thr Gly
                370                 375                 380

GAG CAG ATC GCT GAC GAG CTG ATC CGC CAC TTC GAG GGC GGC GCC GCG      1980
Glu Gln Ile Ala Asp Glu Leu Ile Arg His Phe Glu Gly Gly Ala Ala
            385                 390                 395

GAT GGT TTC ATC ATC TCT CCG GCC TTC CTG CCG GGC TCC TAC GAC GAG      2028
Asp Gly Phe Ile Ile Ser Pro Ala Phe Leu Pro Gly Ser Tyr Asp Glu
        400                 405                 410

TTC GTC GAC CAG GTG GTT CCG GTT CTG CAG GAT CGC GGC TAC TTC CGC      2076
Phe Val Asp Gln Val Val Pro Val Leu Gln Asp Arg Gly Tyr Phe Arg
    415                 420                 425

ACC GAG TAC CAG GGC AAC ACT CTG CGC GAC CAC TTG GGT CTG CGC GTA      2124
Thr Glu Tyr Gln Gly Asn Thr Leu Arg Asp His Leu Gly Leu Arg Val
430                 435                 440                 445

CCA CAA CTG CAA GGA CAA CCT TCA TGACAAGCCG CGTCGACCCC GCAAACCCCG      2178
Pro Gln Leu Gln Gly Gln Pro Ser
                450

GTTCAGAACT CGATTCCGCC ATCCGCGACA CACTGACCTA CAGCAACTGC CCGGTACCCA     2238

ACGCTCTGCT CACGGCATCG GAATCGGGCT TCCTCGACGC CGCCGGCATC GAACTCGACG     2298

TCCTCAGCGG CCAGCAGGGC ACGGTTCATT TCACCTACGA CCAGCCTGCC TACACCCGTT     2358

TTGGGGGTGA GATCCCGCCA CTGCTCAGCG AGGGGTTGCG GGCACCTGGG CGCACGCGTC     2418

TACTCGGCAT CACCCCGCTC TTGGGGCGCC AGGGCTTCTT TGTCCGCGAC GACAGCCCGA     2478

TCACAGCGGC CGCCGACCTT GCCGGACGTC GAATCGGCGT CTCGGCCTCG GCAATTCGCA     2538

TCCTGCGCGG CCAGCTGGGC GACTACCTCG AGTTGGATCC CTGGCGGCAA ACGCTGGTAG     2598

CGCTGGGCTC GTGGGAGGCG CGCGCCTTGT TGCACACCCT TGAGCACGGT GAACTGGGTG     2658

TGGACGACGT CGAGCTGGTG CCGATCAGCA GTCCTGGTGT CGATGTTCCC GCTGAGCAGC     2718

TCGAAGAATC GGCGACCGTC AAGGGTGCGG ACCTCTTTCC CGATGTCGCC CGCGGTCAGG     2778

CCGCGGTGTT GGCCAGCGGA GACGTTGACG CCCTGTACAG TTGGCTGCCC TGGGCCGGGG     2838

AGTTGCAAGC CACCGGGGCC CGCCCAGTGG TGGATCTCGG CCTCGATGAG CGCAATGCCT     2898

ACGCCAGTGT GTGGACGGTC AGCAGCGGGC TGGTTCGCCA GCGACCTGGC CTTGTTCAAC     2958

GACTGGTCGA CGCGGCCGTC GACGCCGGGC TGTGGGCACG CGATCATTCC GACGCGGTGA     3018

CCAGCCTGCA CGCCGCGAAC CTGGGCGTAT CGACCGGAGC AGTAGGCCAG GGCTTCGGCG     3078

CCGACTTCCA GCAGCGTCTG GTTCCACGCC TGGATCACGA CGCCCTCGCC CTCCTGGAGC     3138
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GCACACAGCA ATTCCTGCTC ACCAACAACT TGCTGCAGGA ACCCGTCGCC CTCGATCAGT    3198

GGGCGGCTCC GGAATTTCTG AACAACAGCC TCAATCGCCA CCGATAGGAA CATCCGC       3255

ATG ACA CTG TCA CCT GAA AAG CAG CAC GTT CGA CCA CGC GAC GCC GCC      3303
Met Thr Leu Ser Pro Glu Lys Gln His Val Arg Pro Arg Asp Ala Ala
 1               5                  10                  15

GAC AAC GAT CCC GTC GCG GTT GCC CGT GGG CTA GCC GAA AAG TGG CGA      3351
Asp Asn Asp Pro Val Ala Val Ala Arg Gly Leu Ala Glu Lys Trp Arg
            20                  25                  30

GCC ACC GCC GTC GAG CGT GAT CGC GCC GGG GGT TCG GCA ACA GCC GAG      3399
Ala Thr Ala Val Glu Arg Asp Arg Ala Gly Gly Ser Ala Thr Ala Glu
        35                  40                  45

CGC GAA GAC CTG CGC GCG AGC GCG CTG CTG TCG CTC CTC GTC CCG CGC      3447
Arg Glu Asp Leu Arg Ala Ser Ala Leu Leu Ser Leu Leu Val Pro Arg
    50                  55                  60

GAA TAC GGC GGC TGG GGC GCA GAC TGG CCC ACC GCC ATC GAG GTC GTC      3495
Glu Tyr Gly Gly Trp Gly Ala Asp Trp Pro Thr Ala Ile Glu Val Val
65                  70                  75                  80

CGC GAA ATC GCG GCA GCC GAT GGA TCT TTG GGA CAC CTG TTC GGA TAC      3543
Arg Glu Ile Ala Ala Ala Asp Gly Ser Leu Gly His Leu Phe Gly Tyr
                85                  90                  95

CAC CTC ACC AAC GCC CCG ATG ATC GAA CTG ATC GGC TCG CAG GAA CAA      3591
His Leu Thr Asn Ala Pro Met Ile Glu Leu Ile Gly Ser Gln Glu Gln
            100                 105                 110

GAA GAA CAC CTG TAC ACC CAG ATC GCG CAG AAC AAC TGG TGG ACC GGA      3639
Glu Glu His Leu Tyr Thr Gln Ile Ala Gln Asn Asn Trp Trp Thr Gly
        115                 120                 125

AAT GCC TCC AGC GAG AAC AAC AGC CAC GTG CTG GAC TGG AAG GTC AGC      3687
Asn Ala Ser Ser Glu Asn Asn Ser His Val Leu Asp Trp Lys Val Ser
    130                 135                 140

GCC ACC CCG ACC GAA GAC GGC GGC TAC GTG CTC AAT GGC ACG AAG CAC      3735
Ala Thr Pro Thr Glu Asp Gly Gly Tyr Val Leu Asn Gly Thr Lys His
145                 150                 155                 160

TTC TGC AGC GGC GCC AAG GGG TCG GAC CTG CTG TTC GTG TTC GGC GTC      3783
Phe Cys Ser Gly Ala Lys Gly Ser Asp Leu Leu Phe Val Phe Gly Val
                165                 170                 175

GTC CAG GAT GAT TCT CCG CAG CAG GGT GCG ATC ATT GCT GCC GCT ATC      3831
Val Gln Asp Asp Ser Pro Gln Gln Gly Ala Ile Ile Ala Ala Ala Ile
            180                 185                 190

CCG ACA TCG CGG GCT GGC GTT ACG CCC AAC GAC GAC TGG GCC GCC ATC      3879
Pro Thr Ser Arg Ala Gly Val Thr Pro Asn Asp Asp Trp Ala Ala Ile
        195                 200                 205

GGC ATG CGG CAG ACC GAC AGC GGT TCC ACG GAC TTC CAC AAC GTC AAG      3927
Gly Met Arg Gln Thr Asp Ser Gly Ser Thr Asp Phe His Asn Val Lys
    210                 215                 220

GTC GAG CCT GAC GAA GTG CTG GGC GCG CCC AAC GCC TTC GTT CTC GCC      3975
Val Glu Pro Asp Glu Val Leu Gly Ala Pro Asn Ala Phe Val Leu Ala
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225                 230                 235                 240

TTC ATA CAA TCC GAG CGC GGC AGC CTC TTC GCG CCC ATA GCG CAA TTG      4023
Phe Ile Gln Ser Glu Arg Gly Ser Leu Phe Ala Pro Ile Ala Gln Leu
                245                 250                 255

ATC TTC GCC AAC GTC TAT CTG GGG ATC GCG CAC GGC GCA CTC GAT GCC      4071
Ile Phe Ala Asn Val Tyr Leu Gly Ile Ala His Gly Ala Leu Asp Ala
            260                 265                 270

GCC AGG GAG TAC ACC CGT ACC CAG GCG AGG CCC TGG ACA CCG GCC GGT      4119
Ala Arg Glu Tyr Thr Arg Thr Gln Ala Arg Pro Trp Thr Pro Ala Gly
        275                 280                 285

ATT CAA CAG GCA ACC GAG GAT CCC TAC ACC ATC CGC TCC TAC GGT GAG      4167
Ile Gln Gln Ala Thr Glu Asp Pro Tyr Thr Ile Arg Ser Tyr Gly Glu
    290                 295                 300

TTC ACC ATC GCA TTG CAG GGA GCT GAC GCC GCC GCC CGT GAA GCG GCC      4215
Phe Thr Ile Ala Leu Gln Gly Ala Asp Ala Ala Ala Arg Glu Ala Ala
305                 310                 315                 320

CAC CTG CTG CAG ACG GTG TGG GAC AAG GGC GAC GCG CTC ACC CCC GAG      4263
His Leu Leu Gln Thr Val Trp Asp Lys Gly Asp Ala Leu Thr Pro Glu
                325                 330                 335

GAC CGC GGC GAA CTG ATG GTG AAG GTC TCG GGA GTC AAA GCG TTG GCC      4311
Asp Arg Gly Glu Leu Met Val Lys Val Ser Gly Val Lys Ala Leu Ala
            340                 345                 350

ACC AAC GCC GCC CTC AAC ATC AGC AGC GGC GTC TTC GAG GTG ATC GGC      4359
Thr Asn Ala Ala Leu Asn Ile Ser Ser Gly Val Phe Glu Val Ile Gly
        355                 360                 365

GCG CGC GGA ACA CAT CCC AGG TAC GGT TTC GAC CGC TTC TGG CGC AAC      4407
Ala Arg Gly Thr His Pro Arg Tyr Gly Phe Asp Arg Phe Trp Arg Asn
    370                 375                 380

GTG CGC ACC CAC TCC CTG CAC GAC CCG GTG TCC TAC AAG ATC GCC GAC      4455
Val Arg Thr His Ser Leu His Asp Pro Val Ser Tyr Lys Ile Ala Asp
385                 390                 395                 400

GTC GGC AAG CAC ACC TTG AAC GGT CAA TAC CCG ATT CCC GGC TTC ACC      4503
Val Gly Lys His Thr Leu Asn Gly Gln Tyr Pro Ile Pro Gly Phe Thr
                405                 410                 415

TCC TGAGGATCTG AGGCGCTGAT CGAGGCCGAG GCCACCGCGC GGCCGAGTCG           4556
Ser

CGAATCGCCC GCCGATACTC AGCTTCTCCA TACGTACGGG TGCACACAAG GAGATATTGT    4616

CAAGACCTGT GGATGAGGGT GTTTCAGGCG ACCTCCGTTT CGCTTGATTC GTCGGGCTCA    4676

GCGGGTGAGA TGTCGATGGG TCGTTCGAGC AGCTTGCCTT TGTGGAACAC CGCGCCGGCA    4736

CGGACCAGCG CGACCAGATG GGGGGCGTTG ACCGCCGCCA GCGGGCTTGT GCGGCGTCGA    4796

TCAGCTTGTA GGCCATGGCA ATCCCGCTGC GACGTGACCC AGGGCCCTTG GTGACCTTGG    4856

TTCGCAACCG CACGGTCGCA AACGTCGATT CGATCGGATT CGTAGTGCGC AAGTGGATCC    4916
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AGTGCTCGGC CGGGTACCGG TAGAACTCCA GGAGCACGTC GGCGTCGTCG ACGATCTTGG    4976

CGACCGCCTT GGGGTACTTC GCGCCGTAAT CTACCTCGAA GGCCTTGATC GCGACCTGGG    5036

CCTTGTCGAT GTCCTCGGCG TTGTAGATTT CCCGCATCGC CGCGGTCGCA CCTGGATGAG    5096

CCGACTTGGG CAGCGCAGCA AGCACATTGG CCTGCTTGTG AAACCAGCAG CGCTGTTCAC    5156

GGGTATCCGG AAACACCTCC CGCAGTGCCT TCCAGAACCC CAGCGCCCCA TCACCGACGG    5216

CCAGCACCGG GGCGGTCATC CCGCGGCGTC GGCATGAGCG CAGCAGATCA GCCCACGACT    5276

CTGTGGACTC CCGGAACCCA TCGGTGAGCG CGACGAGCTC CTTGCGGCCG TCGGCGCGGA    5336

CGCCGATCAT CACGAGCAAG CACAGCTTCT CCTGCTCCAG GCGGACATTG AGATGGATGC    5396

CGTCGACCCA TAGGTACACG AAATCGGTGC CCGAGAGATC CCGGTCGGCG AAGGCCTTCG    5456

CCTCGTCCTG CCATTGCGCG GTCAGCCGGG TGATCGTCGA GGCCGACAGC CCGGCACCAG    5516

TGCCGAGGAA CTGCTCCAA                                                 5535
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Thr Gln Gln Arg Gln Met His Leu Ala Gly Phe Phe Ser Ala Gly
 1               5                  10                  15

Asn Val Thr His Ala His Gly Ala Trp Arg His Thr Asp Ala Ser Asn
            20                  25                  30

Asp Phe Leu Ser Gly Lys Tyr Tyr Gln His Ile Ala Arg Thr Leu Glu
        35                  40                  45

Arg Gly Lys Phe Asp Leu Leu Phe Leu Pro Asp Gly Leu Ala Val Glu
    50                  55                  60

Asp Ser Tyr Gly Asp Asn Leu Asp Thr Gly Val Gly Leu Gly Gly Gln
65                  70                  75                  80

Gly Ala Val Ala Leu Glu Pro Ala Ser Val Val Ala Thr Met Ala Ala
                85                  90                  95

Val Thr Glu His Leu Gly Leu Gly Ala Thr Ile Ser Ala Thr Tyr Tyr
            100                 105                 110

Pro Pro Tyr His Val Ala Arg Val Phe Ala Thr Leu Asp Gln Leu Ser
        115                 120                 125

Gly Gly Arg Val Ser Trp Asn Val Val Thr S r Leu Asn Asp Ala Glu
    130                 135                 140

Ala Arg Asn Phe Gly Ile Asn Gln His Leu Glu His Asp Ala Arg Tyr
```

145 150 155 160

Asp Arg Ala Asp Glu Phe Leu Glu Ala Val Lys Lys Leu Trp Asn Ser
165 170 175

Trp Asp Glu Asp Ala Leu Val Leu Asp Lys Ala Ala Gly Val Phe Ala
180 185 190

Asp Pro Ala Lys Val His Tyr Val Asp His His Gly Glu Trp Leu Asn
195 200 205

Val Arg Gly Pro Leu Gln Val Pro Arg Ser Pro Gln Gly Glu Pro Val
210 215 220

Ile Leu Gln Ala Gly Leu Ser Pro Arg Gly Arg Arg Phe Ala Gly Lys
225 230 235 240

Trp Ala Glu Ala Val Phe Ser Leu Ala Pro Asn Leu Glu Val Met Gln
245 250 255

Ala Thr Tyr Gln Gly Ile Lys Ala Glu Val Asp Ala Ala Gly Arg Asp
260 265 270

Pro Asp Gln Thr Lys Ile Phe Thr Ala Val Met Pro Val Leu Gly Glu
275 280 285

Ser Gln Ala Val Ala Gln Glu Arg Leu Glu Tyr Leu Asn Ser Leu Val
290 295 300

His Pro Glu Val Gly Leu Ser Thr Leu Ser Ser His Thr Gly Ile Asn
305 310 315 320

Leu Ala Ala Tyr Pro Leu Asp Thr Pro Ile Lys Asp Ile Leu Arg Asp
325 330 335

Leu Gln Asp Arg Asn Val Pro Thr Gln Leu His Met Phe Ala Ala Ala
340 345 350

Thr His Ser Glu Glu Leu Thr Leu Ala Glu Met Gly Arg Arg Tyr Gly
355 360 365

Thr Asn Val Gly Phe Val Pro Gln Trp Ala Gly Thr Gly Glu Gln Ile
370 375 380

Ala Asp Glu Leu Ile Arg His Phe Glu Gly Gly Ala Ala Asp Gly Phe
385 390 395 400

Ile Ile Ser Pro Ala Phe Leu Pro Gly Ser Tyr Asp Glu Phe Val Asp
405 410 415

Gln Val Val Pro Val Leu Gln Asp Arg Gly Tyr Phe Arg Thr Glu Tyr
420 425 430

Gln Gly Asn Thr Leu Arg Asp His Leu Gly Leu Arg Val Pro Gln Leu
435 440 445

Gln Gly Gln Pro Ser
450

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 416 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Thr Leu Ser Pro Glu Lys Gln His Val Arg Pro Arg Asp Ala Ala
 1               5                  10                  15

Asp Asn Asp Pro Val Ala Val Ala Arg Gly Leu Ala Glu Lys Trp Arg
            20                  25                  30

Ala Thr Ala Val Glu Arg Asp Arg Ala Gly Gly Ser Ala Thr Ala Glu
        35                  40                  45

Arg Glu Asp Leu Arg Ala Ser Ala Leu Leu Ser Leu Leu Val Pro Arg
    50                  55                  60

Glu Tyr Gly Gly Trp Gly Ala Asp Trp Pro Thr Ala Ile Glu Val Val
65                  70                  75                  80

Arg Glu Ile Ala Ala Ala Asp Gly Ser Leu Gly His Leu Phe Gly Tyr
                85                  90                  95

His Leu Thr Asn Ala Pro Met Ile Glu Leu Ile Gly Ser Gln Glu Gln
            100                 105                 110

Glu Glu His Leu Tyr Thr Gln Ile Ala Gln Asn Asn Trp Trp Thr Gly
        115                 120                 125

Asn Ala Ser Ser Glu Asn Asn Ser His Val Leu Asp Trp Lys Val Ser
    130                 135                 140

Ala Thr Pro Thr Glu Asp Gly Gly Tyr Val Leu Asn Gly Thr Lys His
145                 150                 155                 160

Phe Cys Ser Gly Ala Lys Gly Ser Asp Leu Leu Phe Val Phe Gly Val
                165                 170                 175

Val Gln Asp Asp Ser Pro Gln Gln Gly Ala Ile Ile Ala Ala Ala Ile
            180                 185                 190

Pro Thr Ser Arg Ala Gly Val Thr Pro Asn Asp Asp Trp Ala Ala Ile
        195                 200                 205

Gly Met Arg Gln Thr Asp Ser Gly Ser Thr Asp Phe His Asn Val Lys
    210                 215                 220

Val Glu Pro Asp Glu Val Leu Gly Ala Pro Asn Ala Phe Val Leu Ala
225                 230                 235                 240

Phe Ile Gln Ser Glu Arg Gly Ser Leu Phe Ala Pro Ile Ala Gln Leu
                245                 250                 255

Ile Phe Ala Asn Val Tyr Leu Gly Ile Ala His Gly Ala Leu Asp Ala
            260                 265                 270
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Ala Arg Glu Tyr Thr Arg Thr Gln Ala Arg Pro Trp Thr Pro Ala Gly
        275                 280                 285

Ile Gln Gln Ala Thr Glu Asp Pro Tyr Thr Ile Arg Ser Tyr Gly Glu
    290                 295                 300

Phe Thr Ile Ala Leu Gln Gly Ala Asp Ala Ala Ala Arg Glu Ala Ala
305                 310                 315                 320

His Leu Leu Gln Thr Val Trp Asp Lys Gly Asp Ala Leu Thr Pro Glu
                325                 330                 335

Asp Arg Gly Glu Leu Met Val Lys Val Ser Gly Val Lys Ala Leu Ala
            340                 345                 350

Thr Asn Ala Ala Leu Asn Ile Ser Ser Gly Val Phe Glu Val Ile Gly
        355                 360                 365

Ala Arg Gly Thr His Pro Arg Tyr Gly Phe Asp Arg Phe Trp Arg Asn
    370                 375                 380

Val Arg Thr His Ser Leu His Asp Pro Val Ser Tyr Lys Ile Ala Asp
385                 390                 395                 400

Val Gly Lys His Thr Leu Asn Gly Gln Tyr Pro Ile Pro Gly Phe Thr
                405                 410                 415

Ser
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5535 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2148..3245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GCATGCACGT CGCGCCGACG CATTTGCGCG CACGGCTCCG GGCAGTTCTC GCGGCGCTGG      60

AGGCACGGAT GGGCACCCTC AACGAACTCA CCCAAACCAC GCCGATAGCG ATCCTCGCCG     120

AAACCCTCGG CTACAGCCCT CAGACATTGG AAGCTCATGC GCGACGCATC CGGATCGACC     180

TTTGCACGCT ACGTGGCGAC GCGGCTGGAC TGACGCTGGA GGTCCGACCC GACGTGTGTG     240

GTGTAGCGCC GCTTAACGGG TGCGCACGGC GGGACATCGG CCAGCTGGCT TGCCCCTCCT     300

CCGCAGGTAG TCGACCACCC CTTCCCGCAG CGGTCGGAGG TGATCGACCG TTAGGGTCAT     360

TTGCTCGCAG ATCGGCTGAT GTTGCCGATC GACGTGGTCG ACGGGACACG CTCGCGATTG     420
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GCATGGCGTC CGGTGCATAC ACGACGATCT AACCAGATCG ACGGTTTTGA GCGTCGGTCA      480
ACGTCGACTC GATGCGCCGT GCGAGTGAGA TCCTTTGTGG TGCTTGGCTA TTGACCTCGA      540
CAAGGATAGA GATTCGAAGG ACCTCGGATC GACCCAAATG CGGACGGCCG GCAGCGGCGA      600
AGGCGGCCAA GTCATCGGCA CCGTCACCGT CACCTTGACC CGACGTGCCC CGTGGTTCAA      660
GGCCTGAATT TGGCTGGTGG AGCATTGAAA TCAGGTGAAG TTTAACGGTG GGCACACCCC      720
GGGGGTGGGG GTGAGACTGC TTAGCGACAG GAATCTAGCC ATGATTGACA TTTAAAGGAC      780
GCATACGCGA TGACTCAACA ACGACAAATG CATCTGGCCG GTTTCTTCTC GGCCGGCAAT      840
GTGACTCATG CACATGGGGC GTGGCGGCAC ACGGACGCGT CGAATGACTT TCTGTCGGGG      900
AAGTACTACC AACACATCGC CCGTACTCTG GAGCGCGGCA AGTTCGATCT GTTGTTTCTG      960
CCTGACGGGT TGGCCGTCGA GGACAGCTAC GGGGACAACC TGGACACCGG TGTCGGCCTG     1020
GGCGGGCAGG GTGCAGTCGC CTTGGAGCCG GCCAGTGTGG TCGCAACCAT GGCCGCGGTG     1080
ACCGAGCACC TGGGTCTTGG GGCAACCATT TCGGCGACCT ACTATCCCCC GTATCACGTT     1140
GCTCGGGTGT TCGCGACGCT CGATCAGTTG TCAGGGGGTC GGGTGTCCTG GAACGTCGTC     1200
ACCTCGCTCA ACGACGCTGA AGCGCGCAAC TTCGGCATTA ATCAGCATCT GGAACACGAC     1260
GCCCGCTATG ACCGCGCCGA TGAGTTCTTG GAAGCGGTCA AGAAACTCTG GAACAGCTGG     1320
GACGAGGACG CCCTCGTGCT GGACAAGGCG GCCGGCGTGT TCGCCGATCC CGCGAAGGTG     1380
CACTACGTCG ATCACCACGG GGAGTGGCTG AATGTGCGCG GACCTCTGCA GGTACCGCGT     1440
TCACCTCAGG GTGAGCCGGT GATCCTGCAG GCCGGCCTGT CGCCCCGGGG TCGGCGCTTC     1500
GCCGGGAAGT GGGCCGAGGC CGTCTTCAGT CTTGCACCCA ACCTCGAGGT GATGCAGGCC     1560
ACCTACCAGG GCATCAAAGC CGAGGTCGAC GCTGCGGGGC GCGATCCCGA TCAGACGAAA     1620
ATCTTCACCG CCGTGATGCC GGTACTCGGC GAAAGCCAGG CGGTGGCACA GGAACGACTG     1680
GAATATCTCA ACAGTCTGGT CCATCCGGAA GTGGGACTGT CGACGCTATC CAGTCACACC     1740
GGCATCAACC TGGCGGCGTA CCCTCTCGAC ACTCCGATCA AGGACATCCT GCGGGATCTG     1800
CAGGATCGGA ATGTCCCGAC GCAACTGCAC ATGTTCGCCG CCGCAACGCA CAGCGAAGAG     1860
CTCACGCTGG CGGAAATGGG TCGGCGCTAT GGAACCAACG TGGGGTTCGT TCCTCAGTGG     1920
GCCGGTACCG GGGAGCAGAT CGCTGACGAG CTGATCCGCC ACTTCGAGGG CGGCGCCGCG     1980
GATGGTTTCA TCATCTCTCC GGCCTTCCTG CCGGGCTCCT ACGACGAGTT CGTCGACCAG     2040
GTGGTTCCGG TTCTGCAGGA TCGCGGCTAC TTCCGCACCG AGTACCAGGG CAACACTCTG     2100
CGCGACCACT TGGGTCTGCG CGTACCACAA CTGCAAGGAC AACCTTC ATG ACA AGC       2156
                                                    Met Thr Ser
                                                     1

CGC GTC GAC CCC GCA AAC CCC GGT TCA GAA CTC GAT TCC GCC ATC CGC       2204
Arg Val Asp Pro Ala Asn Pro Gly S r Glu Leu Asp Ser Ala Ile Arg
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     5                  10                  15

GAC ACA CTG ACC TAC AGC AAC TGC CCG GTA CCC AAC GCT CTG CTC ACG      2252
Asp Thr Leu Thr Tyr Ser Asn Cys Pro Val Pro Asn Ala Leu Leu Thr
 20                  25                  30                  35

GCA TCG GAA TCG GGC TTC CTC GAC GCC GCC GGC ATC GAA CTC GAC GTC      2300
Ala Ser Glu Ser Gly Phe Leu Asp Ala Ala Gly Ile Glu Leu Asp Val
                 40                  45                  50

CTC AGC GGC CAG CAG GGC ACG GTT CAT TTC ACC TAC GAC CAG CCT GCC      2348
Leu Ser Gly Gln Gln Gly Thr Val His Phe Thr Tyr Asp Gln Pro Ala
             55                  60                  65

TAC ACC CGT TTT GGG GGT GAG ATC CCG CCA CTG CTC AGC GAG GGG TTG      2396
Tyr Thr Arg Phe Gly Gly Glu Ile Pro Pro Leu Leu Ser Glu Gly Leu
         70                  75                  80

CGG GCA CCT GGG CGC ACG CGT CTA CTC GGC ATC ACC CCG CTC TTG GGG      2444
Arg Ala Pro Gly Arg Thr Arg Leu Leu Gly Ile Thr Pro Leu Leu Gly
     85                  90                  95

CGC CAG GGC TTC TTT GTC CGC GAC GAC AGC CCG ATC ACA GCG GCC GCC      2492
Arg Gln Gly Phe Phe Val Arg Asp Asp Ser Pro Ile Thr Ala Ala Ala
100                 105                 110                 115

GAC CTT GCC GGA CGT CGA ATC GGC GTC TCG GCC TCG GCA ATT CGC ATC      2540
Asp Leu Ala Gly Arg Arg Ile Gly Val Ser Ala Ser Ala Ile Arg Ile
                120                 125                 130

CTG CGC GGC CAG CTG GGC GAC TAC CTC GAG TTG GAT CCC TGG CGG CAA      2588
Leu Arg Gly Gln Leu Gly Asp Tyr Leu Glu Leu Asp Pro Trp Arg Gln
            135                 140                 145

ACG CTG GTA GCG CTG GGC TCG TGG GAG GCG CGC GCC TTG TTG CAC ACC      2636
Thr Leu Val Ala Leu Gly Ser Trp Glu Ala Arg Ala Leu Leu His Thr
        150                 155                 160

CTT GAG CAC GGT GAA CTG GGT GTG GAC GAC GTC GAG CTG GTG CCG ATC      2684
Leu Glu His Gly Glu Leu Gly Val Asp Asp Val Glu Leu Val Pro Ile
    165                 170                 175

AGC AGT CCT GGT GTC GAT GTT CCC GCT GAG CAG CTC GAA GAA TCG GCG      2732
Ser Ser Pro Gly Val Asp Val Pro Ala Glu Gln Leu Glu Glu Ser Ala
180                 185                 190                 195

ACC GTC AAG GGT GCG GAC CTC TTT CCC GAT GTC GCC CGC GGT CAG GCC      2780
Thr Val Lys Gly Ala Asp Leu Phe Pro Asp Val Ala Arg Gly Gln Ala
                200                 205                 210

GCG GTG TTG GCC AGC GGA GAC GTT GAC GCC CTG TAC AGT TGG CTG CCC      2828
Ala Val Leu Ala Ser Gly Asp Val Asp Ala Leu Tyr Ser Trp Leu Pro
            215                 220                 225

TGG GCC GGG GAG TTG CAA GCC ACC GGG GCC CGC CCA GTG GTG GAT CTC      2876
Trp Ala Gly Glu Leu Gln Ala Thr Gly Ala Arg Pro Val Val Asp Leu
        230                 235                 240

GGC CTC GAT GAG CGC AAT GCC TAC GCC AGT GTG TGG ACG GTC AGC AGC      2924
Gly Leu Asp Glu Arg Asn Ala Tyr Ala Ser Val Trp Thr Val Ser Ser
    245                 250                 255
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GGG CTG GTT CGC CAG CGA CCT GGC CTT GTT CAA CGA CTG GTC GAC GCG      2972
Gly Leu Val Arg Gln Arg Pro Gly Leu Val Gln Arg Leu Val Asp Ala
260                 265                 270                 275

GCC GTC GAC GCC GGG CTG TGG GCA CGC GAT CAT TCC GAC GCG GTG ACC      3020
Ala Val Asp Ala Gly Leu Trp Ala Arg Asp His Ser Asp Ala Val Thr
                280                 285                 290

AGC CTG CAC GCC GCG AAC CTG GGC GTA TCG ACC GGA GCA GTA GGC CAG      3068
Ser Leu His Ala Ala Asn Leu Gly Val Ser Thr Gly Ala Val Gly Gln
            295                 300                 305

GGC TTC GGC GCC GAC TTC CAG CAG CGT CTG GTT CCA CGC CTG GAT CAC      3116
Gly Phe Gly Ala Asp Phe Gln Gln Arg Leu Val Pro Arg Leu Asp His
        310                 315                 320

GAC GCC CTC GCC CTC CTG GAG CGC ACA CAG CAA TTC CTG CTC ACC AAC      3164
Asp Ala Leu Ala Leu Leu Glu Arg Thr Gln Gln Phe Leu Leu Thr Asn
    325                 330                 335

AAC TTG CTG CAG GAA CCC GTC GCC CTC GAT CAG TGG GCG GCT CCG GAA      3212
Asn Leu Leu Gln Glu Pro Val Ala Leu Asp Gln Trp Ala Ala Pro Glu
340                 345                 350                 355

TTT CTG AAC AAC AGC CTC AAT CGC CAC CGA TAGGAACATC CGCATGACAC        3262
Phe Leu Asn Asn Ser Leu Asn Arg His Arg
                360                 365

TGTCACCTGA AAAGCAGCAC GTTCGACCAC GCGACGCCGC CGACAACGAT CCCGTCGCGG    3322

TTGCCCGTGG GCTAGCCGAA AAGTGGCGAG CCACCGCCGT CGAGCGTGAT CGCGCCGGGG    3382

GTTCGGCAAC AGCCGAGCGC GAAGACCTGC GCGCGAGCGC GCTGCTGTCG CTCCTCGTCC    3442

CGCGCGAATA CGGCGGCTGG GGCGCAGACT GGCCCACCGC CATCGAGGTC GTCCGCGAAA    3502

TCGCGGCAGC CGATGGATCT TTGGGACACC TGTTCGGATA CCACCTCACC AACGCCCCGA    3562

TGATCGAACT GATCGGCTCG CAGGAACAAG AAGAACACCT GTACACCCAG ATCGCGCAGA    3622

ACAACTGGTG GACCGGAAAT GCCTCCAGCG AGAACAACAG CCACGTGCTG GACTGGAAGG    3682

TCAGCGCCAC CCCGACCGAA GACGGCGGCT ACGTGCTCAA TGGCACGAAG CACTTCTGCA    3742

GCGGCGCCAA GGGGTCGGAC CTGCTGTTCG TGTTCGGCGT CGTCCAGGAT GATTCTCCGC    3802

AGCAGGGTGC GATCATTGCT GCCGCTATCC CGACATCGCG GGCTGGCGTT ACGCCCAACG    3862

ACGACTGGGC CGCCATCGGC ATGCGGCAGA CCGACAGCGG TTCCACGGAC TTCCACAACG    3922

TCAAGGTCGA GCCTGACGAA GTGCTGGGCG CGCCCAACGC CTTCGTTCTC GCCTTCATAC    3982

AATCCGAGCG CGGCAGCCTC TTCGCGCCCA TAGCGCAATT GATCTTCGCC AACGTCTATC    4042

TGGGGATCGC GCACGGCGCA CTCGATGCCG CCAGGGAGTA CACCCGTACC CAGGCGAGGC    4102

CCTGGACACC GGCCGGTATT CAACAGGCAA CCGAGGATCC CTACACCATC CGCTCCTACG    4162

GTGAGTTCAC CATCGCATTG CAGGGAGCTG ACGCCGCCGC CCGTGAAGCG GCCCACCTGC    4222

TGCAGACGGT GTGGGACAAG GGCGACGCGC TCACCCCCGA GGACCGCGGC GAACTGATGG    4282
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TGAAGGTCTC GGGAGTCAAA GCGTTGGCCA CCAACGCCGC CCTCAACATC AGCAGCGGCG 4342

TCTTCGAGGT GATCGGCGCG CGCGGAACAC ATCCCAGGTA CGGTTTCGAC CGCTTCTGGC 4402

GCAACGTGCG CACCCACTCC CTGCACGACC CGGTGTCCTA CAAGATCGCC GACGTCGGCA 4462

AGCACACCTT GAACGGTCAA TACCCGATTC CCGGCTTCAC CTCCTGAGGA TCTGAGGCGC 4522

TGATCGAGGC CGAGGCCACC GCGCGGCCGA GTCGCGAATC GCCCGCCGAT ACTCAGCTTC 4582

TCCATACGTA CGGGTGCACA CAAGGAGATA TTGTCAAGAC CTGTGGATGA GGGTGTTTCA 4642

GGCGACCTCC GTTTCGCTTG ATTCGTCGGG CTCAGCGGGT GAGATGTCGA TGGGTCGTTC 4702

GAGCAGCTTG CCTTTGTGGA ACACCGCGCC GGCACGGACC AGCGCGACCA GATGGGGGGC 4762

GTTGACCGCC GCCAGCGGGC TTGTGCGGCG TCGATCAGCT TGTAGGCCAT GGCAATCCCG 4822

CTGCGACGTG ACCCAGGGCC CTTGGTGACC TTGGTTCGCA ACCGCACGGT CGCAAACGTC 4882

GATTCGATCG GATTCGTAGT GCGCAAGTGG ATCCAGTGCT CGGCCGGGTA CCGGTAGAAC 4942

TCCAGGAGCA CGTCGGCGTC GTCGACGATC TTGGCGACCG CCTTGGGGTA CTTCGCGCCG 5002

TAATCTACCT CGAAGGCCTT GATCGCGACC TGGGCCTTGT CGATGTCCTC GGCGTTGTAG 5062

ATTTCCCGCA TCGCCGCGGT CGCACCTGGA TGAGCCGACT TGGGCAGCGC AGCAAGCACA 5122

TTGGCCTGCT TGTGAAACCA GCAGCGCTGT TCACGGGTAT CCGGAAACAC CTCCCGCAGT 5182

GCCTTCCAGA ACCCCAGCGC CCCATCACCG ACGGCCAGCA CCGGGGCGGT CATCCCGCGG 5242

CGTCGGCATG AGCGCAGCAG ATCAGCCCAC GACTCTGTGG ACTCCCGGAA CCCATCGGTG 5302

AGCGCGACGA GCTCCTTGCG GCCGTCGGCG CGGACGCCGA TCATCACGAG CAAGCACAGC 5362

TTCTCCTGCT CCAGGCGGAC ATTGAGATGG ATGCCGTCGA CCCATAGGTA CACGAAATCG 5422

GTGCCCGAGA GATCCCGGTC GGCGAAGGCC TTCGCCTCGT CCTGCCATTG CGCGGTCAGC 5482

CGGGTGATCG TCGAGGCCGA CAGCCCGGCA CCAGTGCCGA GGAACTGCTC CAA 5535

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 365 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Thr Ser Arg Val Asp Pro Ala Asn Pro Gly Ser Glu Leu Asp Ser
1 5 10 15

Ala Ile Arg Asp Thr Leu Thr Tyr Ser Asn Cys Pro Val Pro Asn Ala
20 25 30

Leu L u Thr Ala Ser Glu S r Gly Phe Leu Asp Ala Ala Gly Ile Glu
35 40 45

```
Leu Asp Val Leu Ser Gly Gln Gln Gly Thr Val His Phe Thr Tyr Asp
     50                  55                  60

Gln Pro Ala Tyr Thr Arg Phe Gly Gly Glu Ile Pro Pro Leu Leu Ser
 65                  70                  75                  80

Glu Gly Leu Arg Ala Pro Gly Arg Thr Arg Leu Leu Gly Ile Thr Pro
                 85                  90                  95

Leu Leu Gly Arg Gln Gly Phe Phe Val Arg Asp Asp Ser Pro Ile Thr
            100                 105                 110

Ala Ala Ala Asp Leu Ala Gly Arg Arg Ile Gly Val Ser Ala Ser Ala
        115                 120                 125

Ile Arg Ile Leu Arg Gly Gln Leu Gly Asp Tyr Leu Glu Leu Asp Pro
    130                 135                 140

Trp Arg Gln Thr Leu Val Ala Leu Gly Ser Trp Glu Ala Arg Ala Leu
145                 150                 155                 160

Leu His Thr Leu Glu His Gly Glu Leu Gly Val Asp Asp Val Glu Leu
                165                 170                 175

Val Pro Ile Ser Ser Pro Gly Val Asp Val Pro Ala Glu Gln Leu Glu
            180                 185                 190

Glu Ser Ala Thr Val Lys Gly Ala Asp Leu Phe Pro Asp Val Ala Arg
        195                 200                 205

Gly Gln Ala Ala Val Leu Ala Ser Gly Asp Val Asp Ala Leu Tyr Ser
    210                 215                 220

Trp Leu Pro Trp Ala Gly Glu Leu Gln Ala Thr Gly Ala Arg Pro Val
225                 230                 235                 240

Val Asp Leu Gly Leu Asp Glu Arg Asn Ala Tyr Ala Ser Val Trp Thr
                245                 250                 255

Val Ser Ser Gly Leu Val Arg Gln Arg Pro Gly Leu Val Gln Arg Leu
            260                 265                 270

Val Asp Ala Ala Val Asp Ala Gly Leu Trp Ala Arg Asp His Ser Asp
        275                 280                 285

Ala Val Thr Ser Leu His Ala Ala Asn Leu Gly Val Ser Thr Gly Ala
    290                 295                 300

Val Gly Gln Gly Phe Gly Ala Asp Phe Gln Gln Arg Leu Val Pro Arg
305                 310                 315                 320

Leu Asp His Asp Ala Leu Ala Leu Leu Glu Arg Thr Gln Gln Phe Leu
                325                 330                 335

Leu Thr Asn Asn Leu Leu Gln Glu Pro Val Ala Leu Asp Gln Trp Ala
            340                 345                 350

Ala Pro Glu Phe Leu Asn Asn Ser Leu Asn Arg His Arg
        355                 360                 365
```

CLAIMS

1. A recombinant DNA molecule containing a gene which encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

2. The recombinant DNA molecule of Claim 1 wherein said fossil fuel is petroleum.

3. A recombinant DNA molecule containing a gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

4. The recombinant DNA molecule of Claim 3 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

5. The recombinant DNA molecule of Claim 3 wherein said fossil fuel is petroleum.

6. Purified DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

7. Purified DNA of Claim 6 wherein said DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

8. Purified DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing petroleum which contains organic sulfur molecules.

9. Purified DNA of Claim 8 wherein said DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

10. A recombinant DNA vector containing a DNA molecule containing a gene which encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

11. A recombinant DNA vector containing a DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

12. The recombinant DNA vector of Claim 11 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

13. A DNA plasmid vector containing a DNA derived from a microorganism wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

14. A DNA plasmid vector containing a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

15. The DNA plasmid vector of Claim 14 wherein the DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

16. A DNA plasmid containing a recombinant DNA molecule containing the gene of a microorganism wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

17. A DNA plasmid containing a recombinant DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

18. The DNA plasmid of Claim 17 wherein said gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

19. A DNA plasmid containing an isolated DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

20. The DNA plasmid of Claim 19 wherein the isolated DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

21. Plasmid pTOXI-1.

22. Plasmid pTOXI-2.

23. A microorganism containing a recombinant DNA plasmid containing a gene which expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

24. A microorganism containing a recombinant DNA plasmid containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

25. The microorganism of Claim 24 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

26. A microorganism containing a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said transformed microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

27. The microorganism of Claim 26 wherein the DNA plasmid is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

28. Complemented host organism CPE-648 containing plasmid pTOXI-1.

29. Complemented host organism CPE-648 containing plasmid pTOXI-2.

30. A method of desulfurizing a fossil fuel which contains organic sulfur molecules through the use of an organism containing a recombinant DNA plasmid containing a gene which expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules, comprising the steps of:

a) contacting the fossil fuel with the microorganism; and

b) incubating the fossil fuel and microorganism mixture under conditions sufficient to bring about the catalytic cleavage of organic carbon-sulfur bonds,

whereby the organic sulfur content of the fossil fuel is significantly reduced.

31. A method of desulfurizing a fossil fuel which contains organic sulfur molecules through the use of a microorganism containing a recombinant DNA plasmid containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules, comprising the steps of:

a) contacting the fossil fuel with the microorganism; and

b) incubating the fossil fuel and microorganism mixture under conditions sufficient to bring about the catalytic cleavage of organic carbon-sulfur bonds,

whereby the organic sulfur content of the fossil fuel is significantly reduced.

32. The transformed microorganism of Claim 31 wherein the gene is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

33. A method of desulfurizing a fossil fuel which contains organic sulfur molecules through the use of a microorganism containing a DNA plasmid containing DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said microorganism expresses a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules, comprising the steps of:

a) contacting the fossil fuel with said microorganism; and

b) incubating the fossil fuel and transformed microorganism mixture under conditions sufficient to bring about the catalytic cleavage of organic carbon-sulfur bonds,

whereby the organic sulfur content of the fossil fuel is significantly reduced.

34. The method of desulfurizing a fossil fuel of Claim 33 wherein the fossil fuel is petroleum.

35. The method of desulfurizing a fossil fuel of Claim 33 wherein the DNA is derived from the IGTS8 strain of Rhodococcus rhodochrous bacteria.

36. A nucleic acid probe capable of hybridizing to all or a portion of a recombinant DNA molecule containing the gene of a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

37. A nucleic acid probe capable of hybridizing to all or a portion of a recombinant DNA molecule containing the gene of a strain of Rhodococcus rhodochrous bacteria wherein said gene encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

38. A nucleic acid probe capable of hybridizing to a all or a portion of a DNA derived from a strain of Rhodococcus rhodochrous bacteria wherein said DNA encodes a biocatalyst capable of desulfurizing a fossil fuel which contains organic sulfur molecules.

39. The nucleic acid sequence set forth in Sequence No. 1.

40. The nucleic acid sequence set forth in Sequence No. 2.

41. The nucleic acid sequence set forth in Sequence No. 3.

42. The nucleic acid sequence set forth in Sequence No. 4.

43. The nucleic acid sequence set forth in Sequence No. 5.

44. The DNA sequence for ORF-1.

45. The DNA sequence for ORF-2.

46. The DNA sequence for ORF-3.

47. The amino acid sequence for ORF-1.

48. The amino acid sequence for ORF-2.

49. The amino acid sequence for ORF-3.

50. The DNA sequences set forth starting on page 66 through page 80.

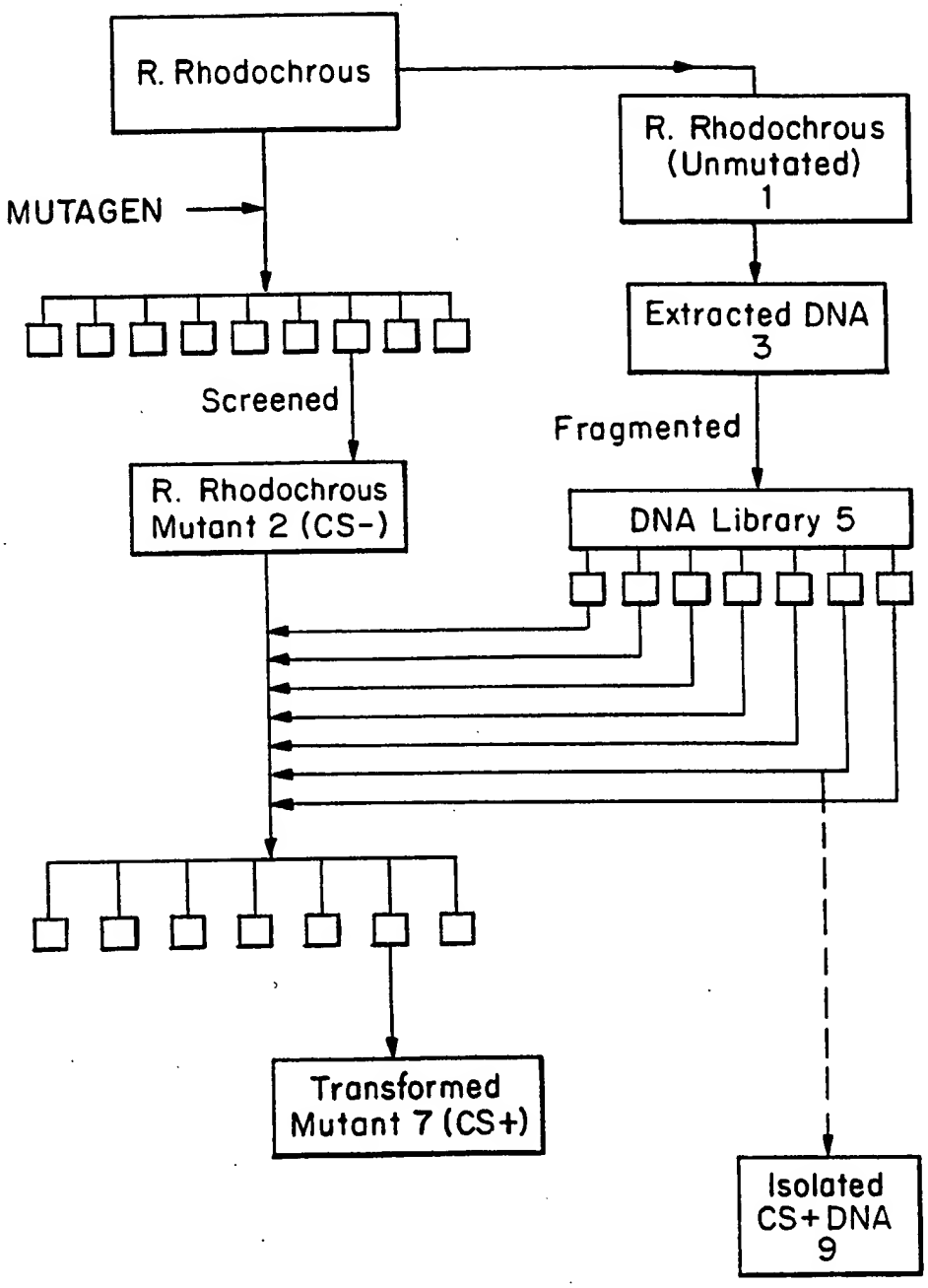
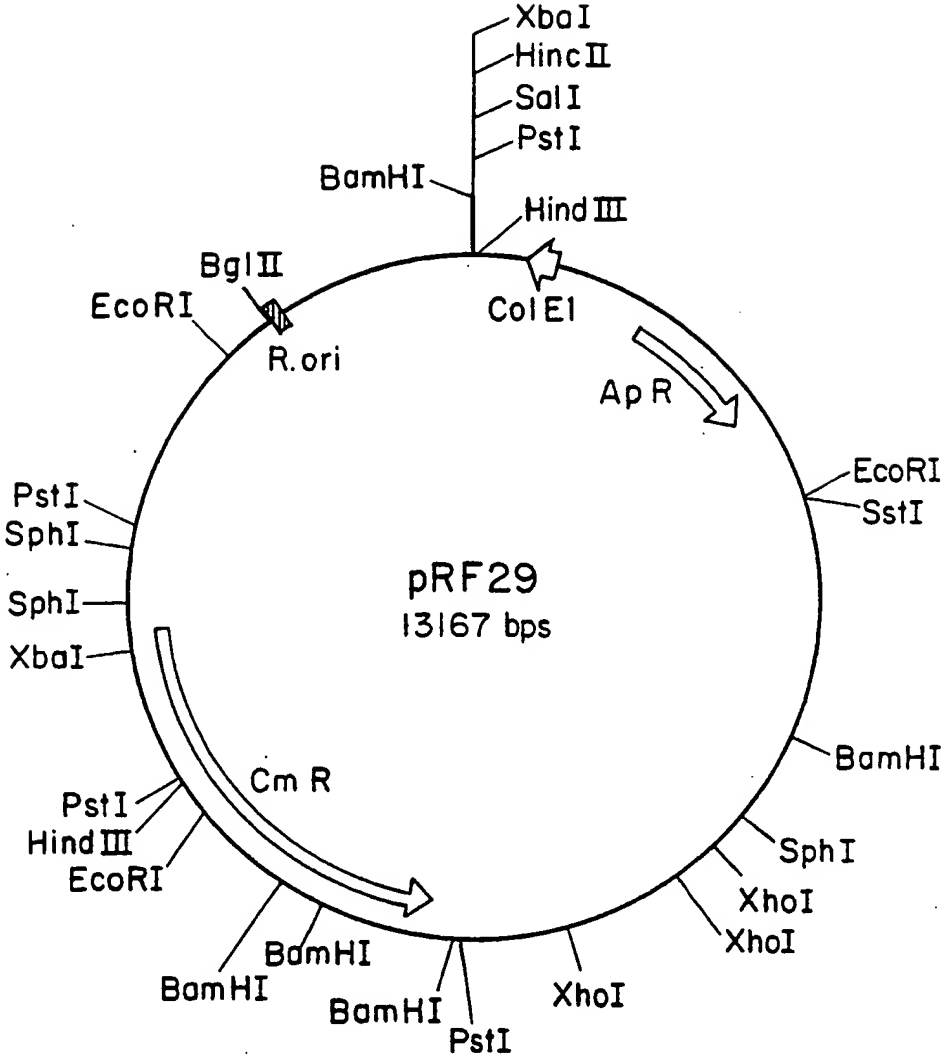

R. Rhodochrous
R. Rhodochrous (Unmutated) 1
MUTAGEN
Extracted DNA 3
Screened
Fragmented
R. Rhodochrous Mutant 2 (CS-)
DNA Library 5
Transformed Mutant 7 (CS+)
Isolated CS+ DNA 9

FIG. 1


XbaI
HincII
SalI
PstI
BamHI
HindIII
BglII
EcoRI
R.ori
ColE1
ApR
EcoRI
SstI
PstI
SphI
SphI
XbaI
pRF29
13167 bps
BamHI
SphI
XhoI
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BamHI
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EcoRI
Cm R

FIG. 2

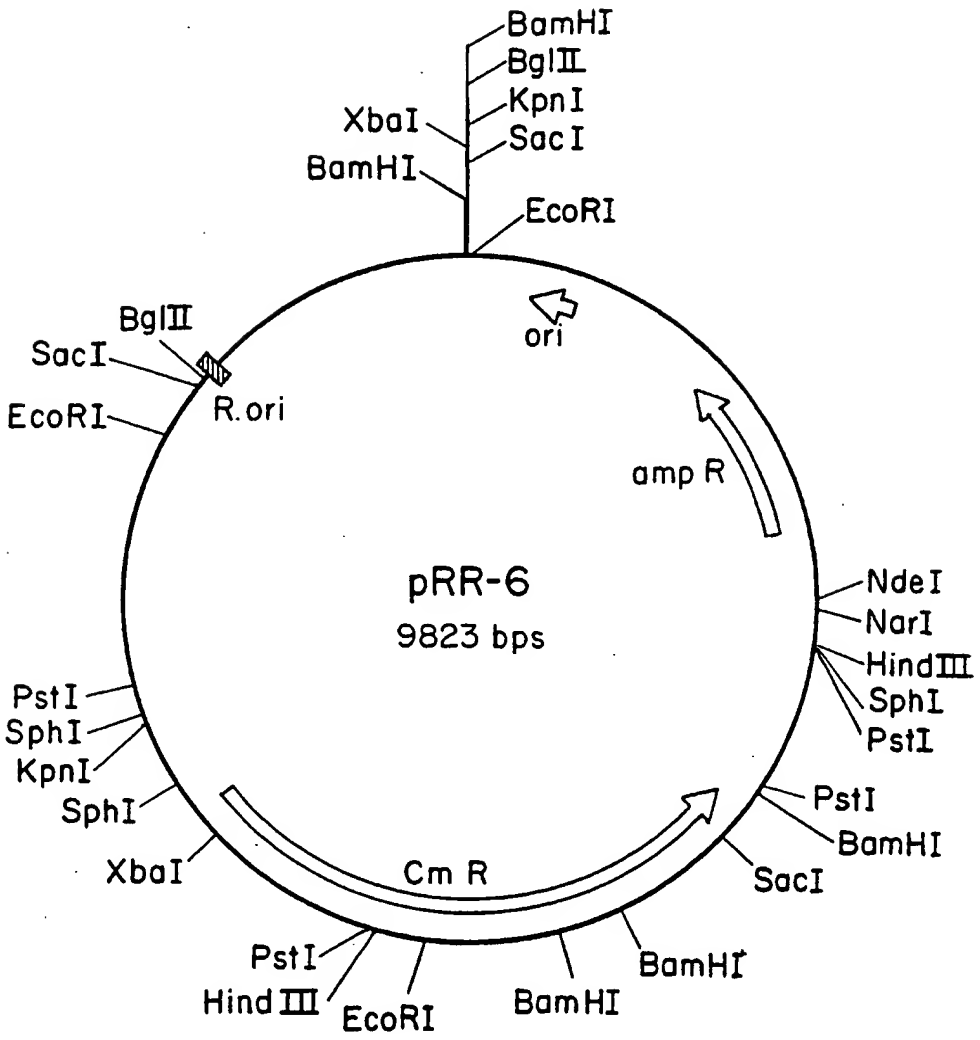
BamHI
BglII
KpnI
SacI
XbaI
BamHI
EcoRI
ori
BglII
SacI
EcoRI
R.ori
amp R
pRR-6
9823 bps
NdeI
NarI
HindIII
SphI
PstI
PstI
SphI
KpnI
SphI
PstI
BamHI
SacI
XbaI
Cm R
PstI
HindIII
EcoRI
BamHI
BamHI

FIG. 3

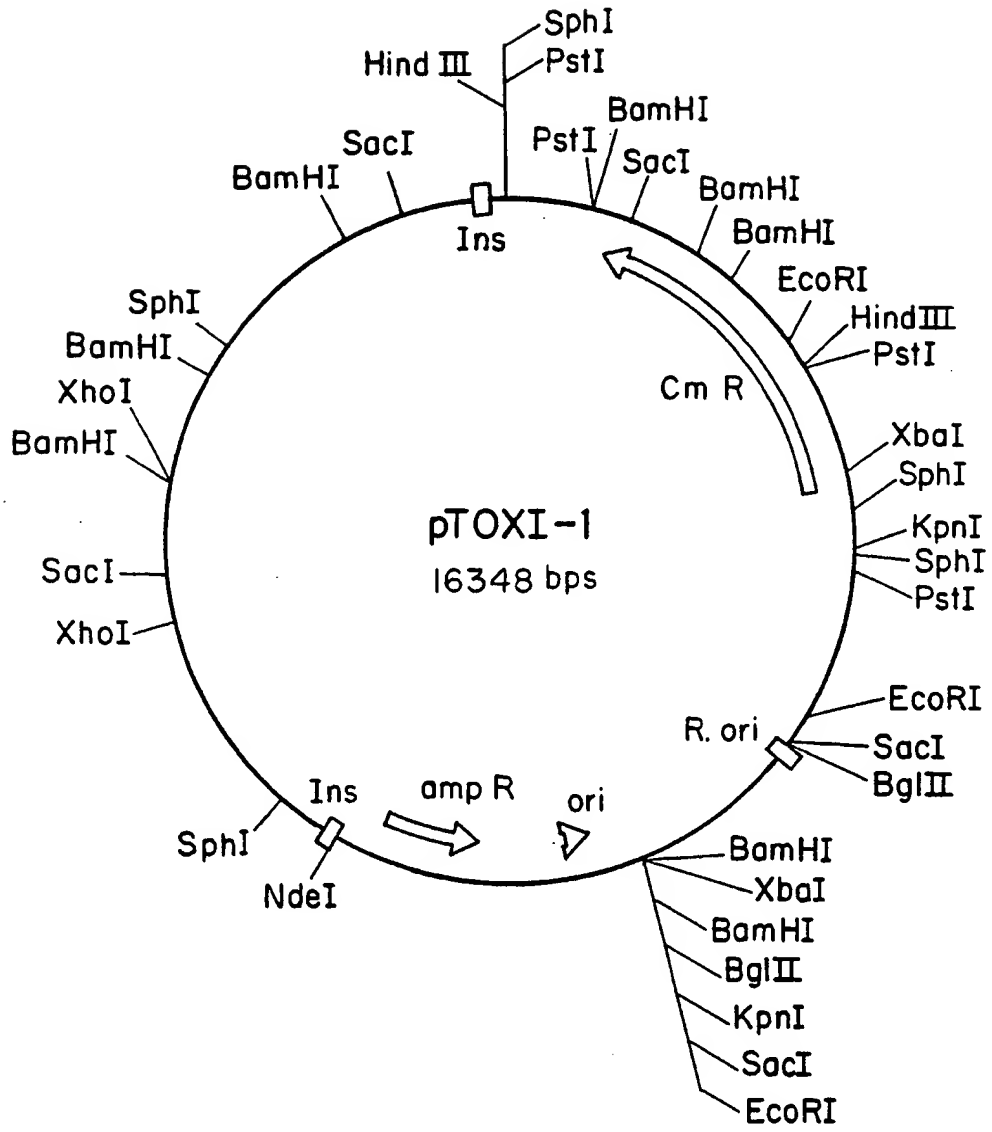
SphI
Hind III
PstI
BamHI
PstI
SacI
SacI
BamHI
BamHI
Ins
EcoRI
HindIII
PstI
Cm R
SphI
BamHI
XhoI
BamHI
XbaI
SphI
KpnI
SphI
PstI
pTOXI-1
16348 bps
SacI
XhoI
EcoRI
R. ori
SacI
BglII
Ins
amp R
ori
SphI
NdeI
BamHI
XbaI
BamHI
BglII
KpnI
SacI
EcoRI

FIG. 4

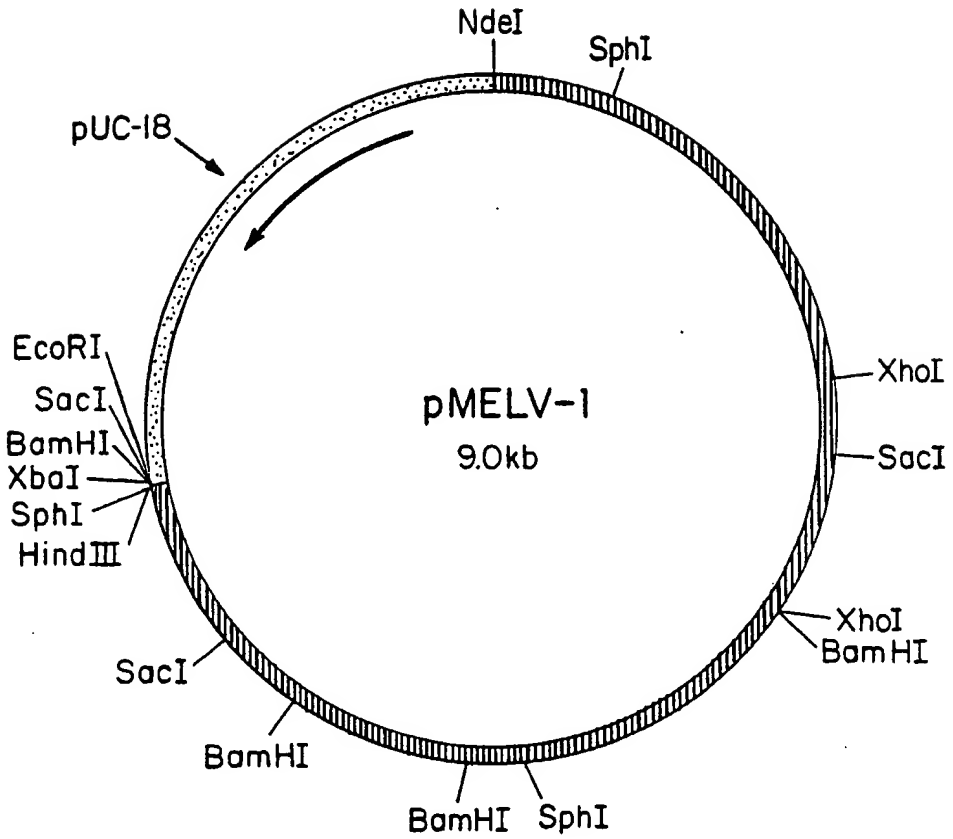
NdeI
SphI
pUC-18
EcoRI
SacI
BamHI
XbaI
SphI
HindIII
pMELV-1
9.0kb
XhoI
SacI
XhoI
BamHI
SacI
BamHI
BamHI
SphI

FIG. 5

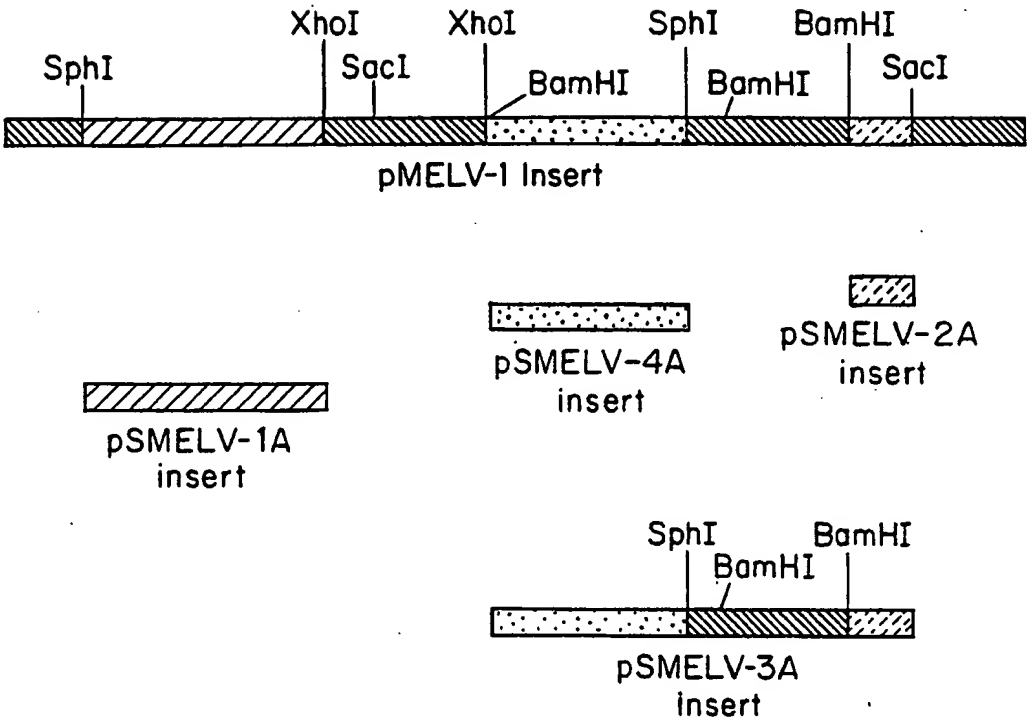
XhoI
XhoI
SphI
BamHI
SphI
SacI
BamHI
BamHI
SacI
pMELV-1 Insert
pSMELV-4A
insert
pSMELV-2A
insert
pSMELV-1A
insert
SphI
BamHI
BamHI
pSMELV-3A
insert

FIG. 6

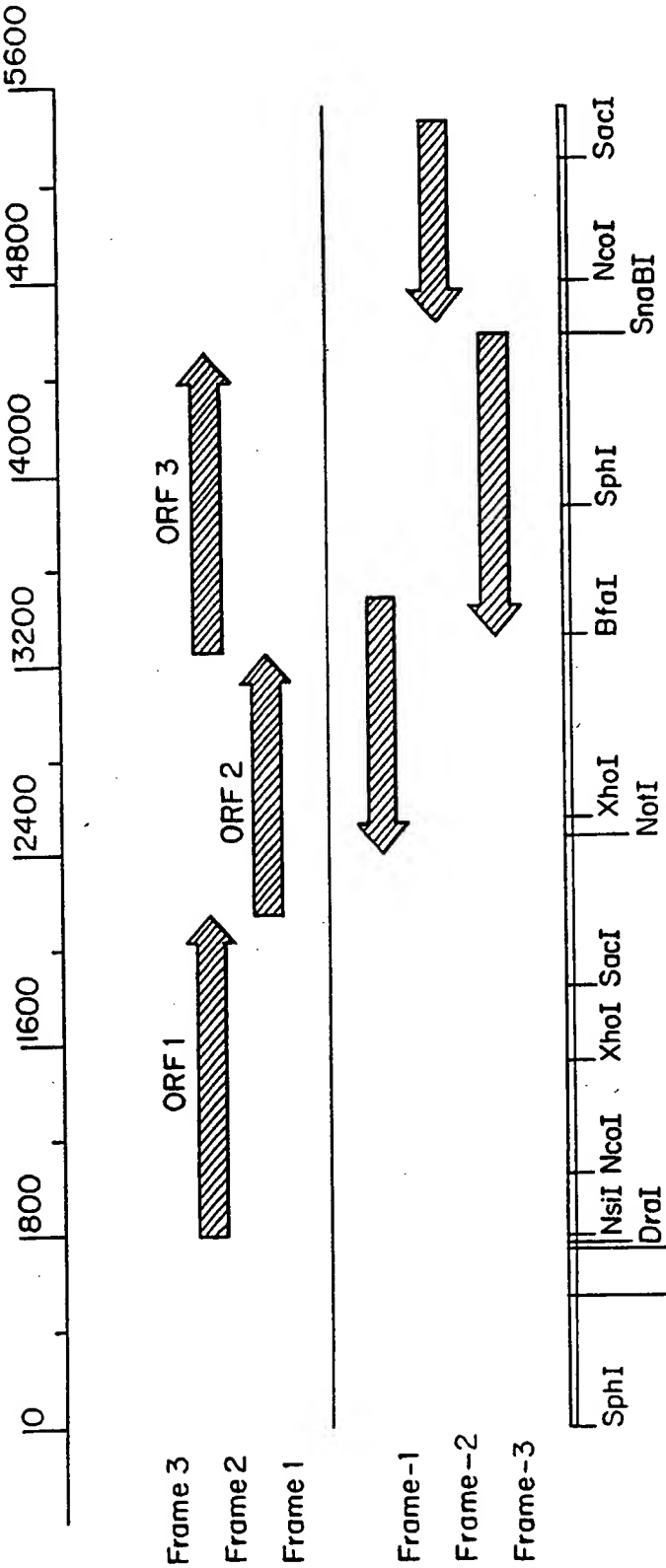
0
800
1600
2400
3200
4000
4800
5600
ORF 1
ORF 2
ORF 3
Frame 3
Frame 2
Frame 1
Frame-1
Frame-2
Frame-3
SphI
NsiI
NcoI
DraI
BfaI
BstBI
XhoI
SacI
XhoI
NotI
BfaI
SphI
NcoI
SnaBI
SacI

FIG. 7

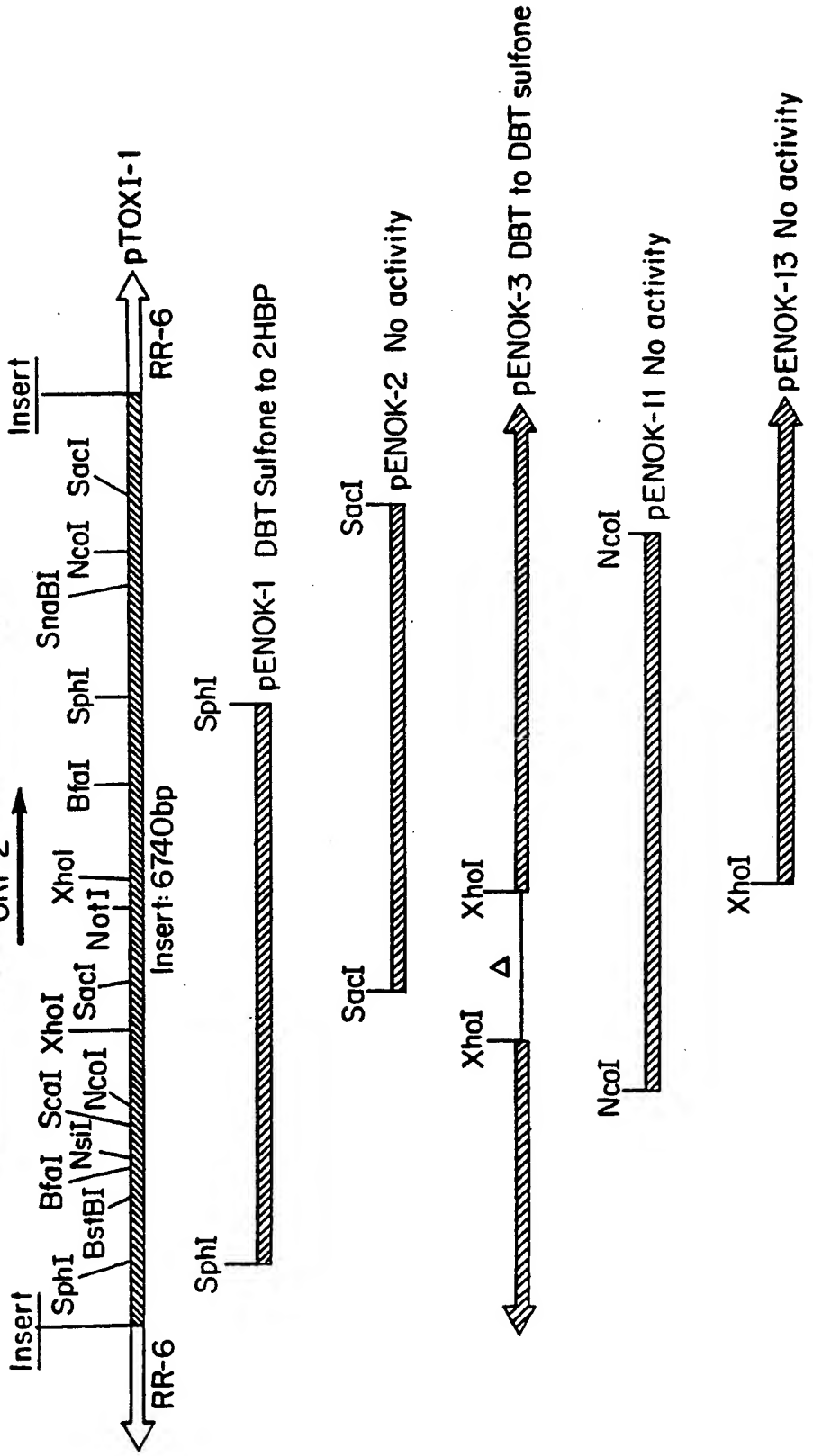
ORF 1
ORF 2
ORF 3
Insert
SphI
BstBI
BfaI
NsiI
ScaI
NcoI
XhoI
SacI
NotI
Insert: 6740bp
BfaI
SphI
SnaBI
NcoI
SacI
RR-6
pTOX1-1
SphI
pENOK-1 DBT Sulfone to 2HBP
SacI
pENOK-2 No activity
XhoI
Δ
XhoI
pENOK-3 DBT to DBT sulfone
NcoI
pENOK-11 No activity
XhoI
pENOK-13 No activity

FIG. 8A

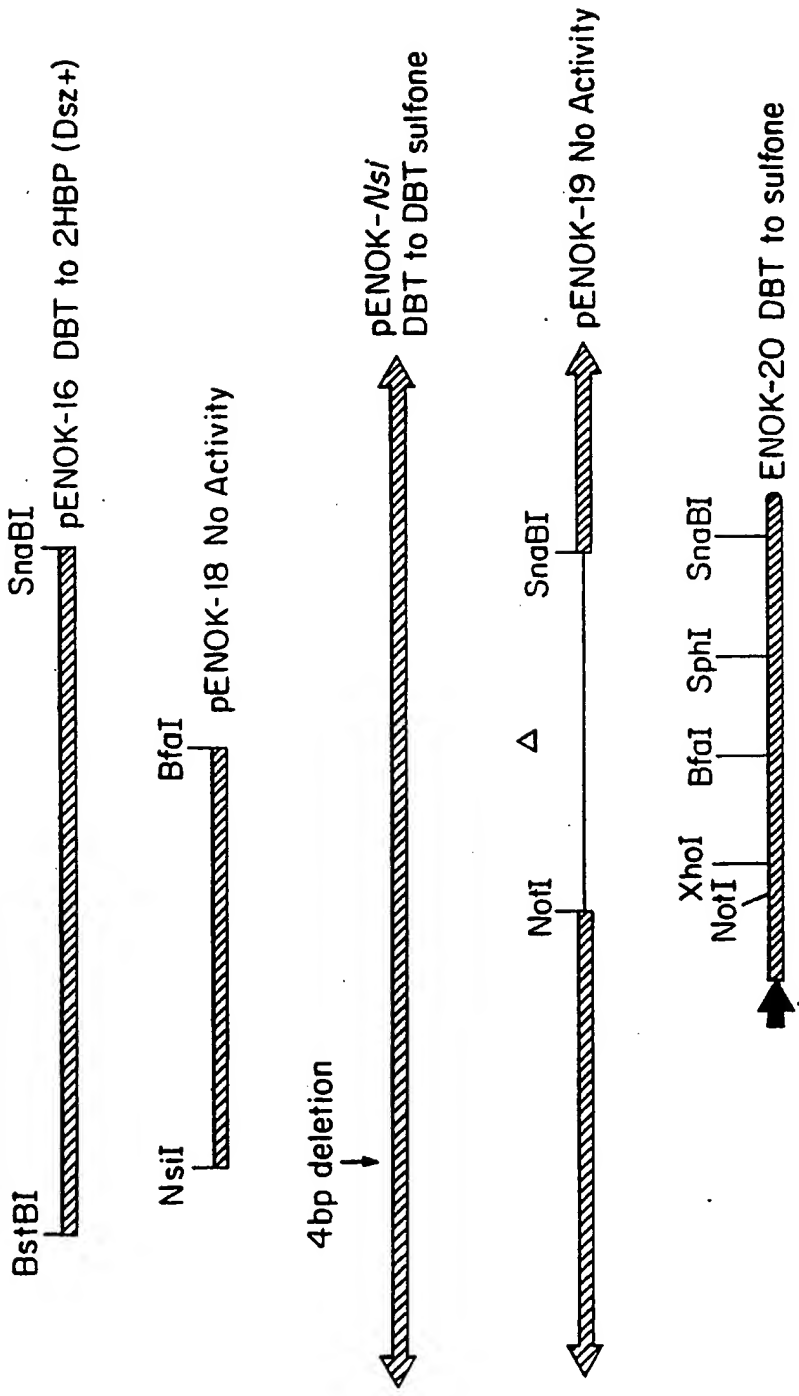
BstBI
SnaBI
pENOK-16 DBT to 2HBP (Dsz+)
NsiI
BfaI
pENOK-18 No Activity
4bp deletion
pENOK-Nsi
DBT to DBT sulfone
NotI
Δ
SnaBI
pENOK-19 No Activity
XhoI
NotI
BfaI
SphI
SnaBI
ENOK-20 DBT to sulfone
Chloramphenicol
Resistance Gene
Promoter

FIG. 8B

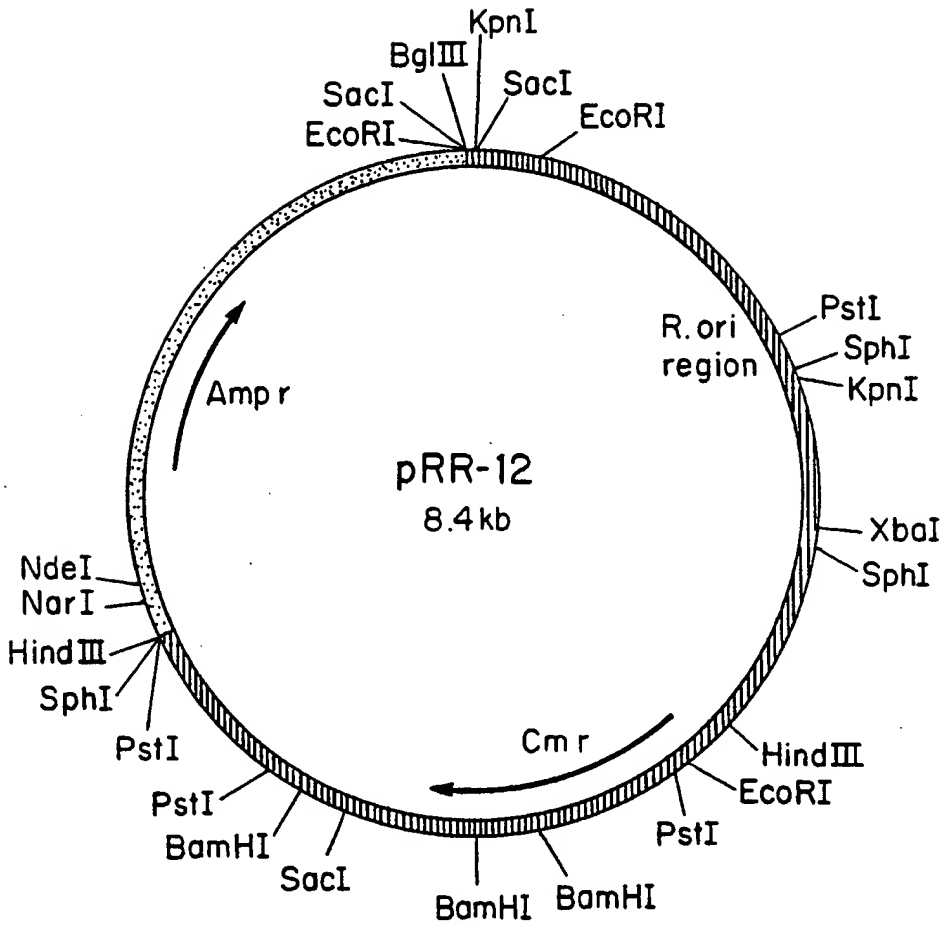
KpnI
BglIII
SacI
SacI
EcoRI
EcoRI
PstI
R. ori
region
SphI
KpnI
Amp r
pRR-12
8.4 kb
XbaI
SphI
NdeI
NarI
HindIII
SphI
PstI
Cm r
HindIII
EcoRI
PstI
PstI
BamHI
SacI
BamHI
BamHI

FIG. 9

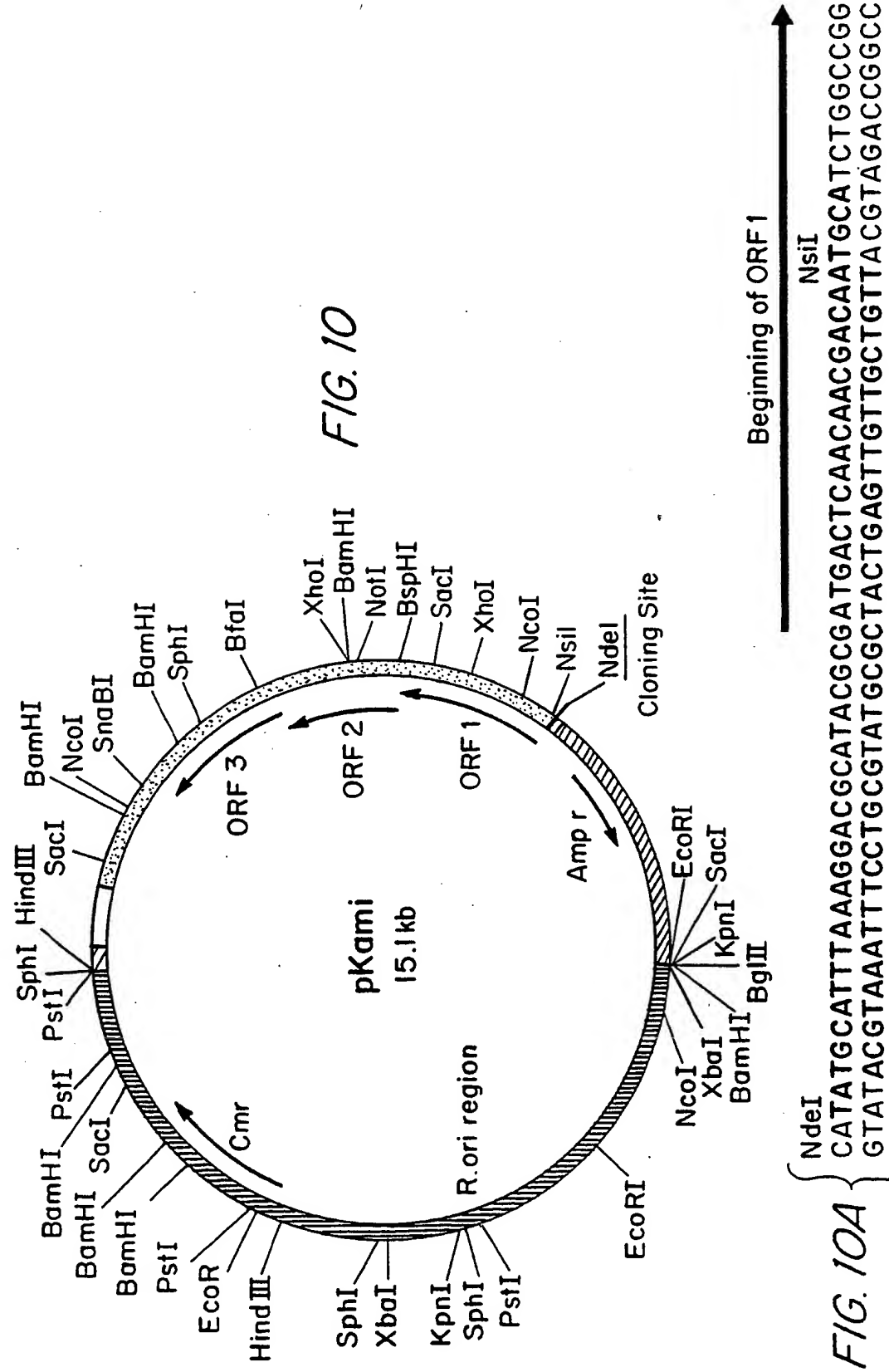

FIG. 10

Beginning of ORF 1

NsiI

NdeI
CATATGCATTTAAAGGACGCATACGCGATGACTCAACAACGACAATGCATCTGGCCGG
GTATACGTAAATTTCCTGCGTATGCGCTACTGAGTTGTTGCTGTTACGTAGACCGGCC

(Synthetic Linkers in bold)

FIG. 10A

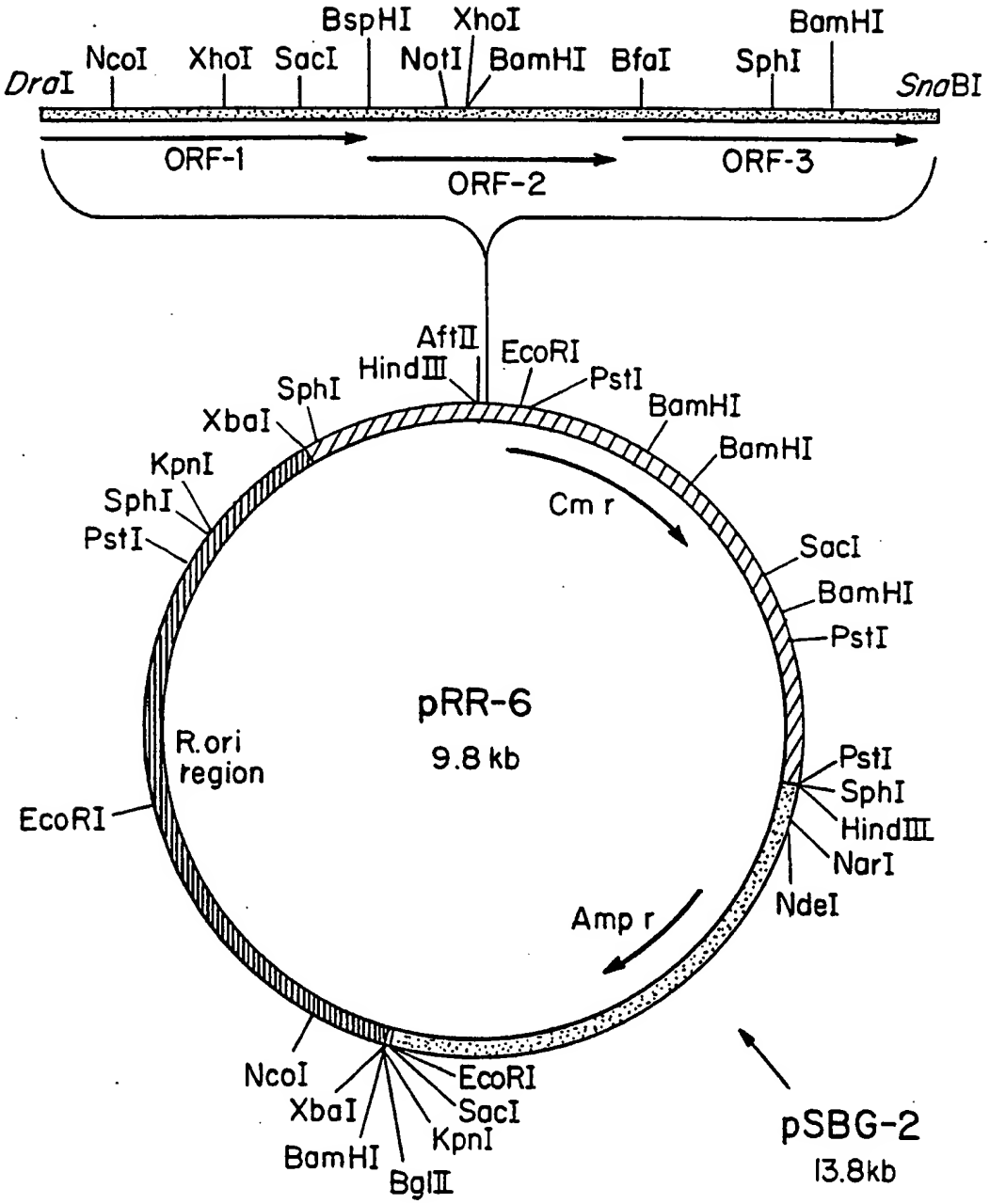
BspHI
XhoI
BamHI
NcoI
XhoI
SacI
NotI
BamHI
BfaI
SphI
DraI
SnoBI
ORF-1
ORF-2
ORF-3
AftII
HindIII
EcoRI
PstI
SphI
BamHI
XbaI
BamHI
KpnI
SphI
Cm r
PstI
SacI
BamHI
PstI
pRR-6
9.8 kb
R.ori
region
PstI
SphI
HindIII
NarI
EcoRI
NdeI
Amp r
NcoI
EcoRI
XbaI
SacI
KpnI
BamHI
BglII
pSBG-2
13.8kb

FIG. 11

INTERNATIONAL SEARCH REPORT

Inter mal Application No
PCT/US 93/06497

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/52 C07K15/00 C10G32/00 C12S1/02 C12N1/21
//(C12N1/21,C12R1:01,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07K C10G C12R C12S

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 445 896 (INSTITUTE OF GAS TECHNOLOGY) 11 September 1991 see page 12, line 17 - line 23 ---	30-35
Y	TRENDS IN BIOTECHNOLOGY vol. 7, no. 4 , April 1989 , ELSEVIER SCIENCE PUBLISHERS,LTD.,CAMBRIDGE,UK; pages 97 - 101 J.J. KILBANE 'Desulfurization of coal: the microbial solution' cited in the application see page 100, middle column, line 5 - right column, line 9 ---	30-35
P,Y	WO,A,92 16602 (ENVIRONEMENTAL BIOSCIENCE CORPORATION) 1 October 1992 see page 20, line 22 - page 21, line 3 ---	30-35
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1993	07. 12. 93
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer HORNIG, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte: nal Application No
PCT/US 93/06497

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO,A,92 19700 (ENERGY BIOSYSTEM CORPORATION) 12 November 1992 see page 18, line 4 - line 15	30-35

1

INTERNATIONAL SEARCH REPORT

ormation on patent family members

Intern· al Application No
PCT/US 93/06497

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0445896	11-09-91	US-A-	5132219	21-07-92
WO-A-9216602	01-10-92	US-A-	5232854	03-08-93
		AU-A-	1643992	21-10-92
		CN-A-	1064880	30-09-92
WO-A-9219700	12-11-92	AU-A-	2233992	21-12-92
		CN-A-	1066285	18-11-92